

# **NEW ASSAYS FOR BIOLOGICALLY ACTIVE QUINONES**

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## ABSTRACT

This thesis investigates the development of an HPLC assay for vitamin K, and the synthesis of carboxylic acid functionalised analogues of vitamin K and coenzyme Q. These analogues were used to investigate the feasibility of an immunoassay based method for the measurement of vitamin K.

Chapter one outlines the biochemistry of vitamin K and coenzyme Q. The biological functions of these quinones and their proposed role in many diverse disease states are discussed.

Chapter two contains a review of literature methods for the assay of vitamin K in plasma, food and tissue samples. The development of an HPLC assay for vitamin K using a metal catalyst, in the presence of an alcohol, to effect reduction of the quinone to the fluorescent hydroquinone for detection is presented. An investigation of a number of metal catalyst/alcohol reduction systems showed platinum black/methanol to give the largest fluorescent response. The generality of this system towards a series of quinones showed that naphthoquinones (including vitamin K<sub>1</sub>, vitamin K<sub>3</sub>, 1,4-naphthoquinone and 2-hydroxy-1,4-naphthoquinone) were efficiently reduced, while the reduction of various benzoquinones (including CoQ-10 and CoQ-0) was also achieved. This chemistry was then applied to the measurement of vitamin K in both standard solutions and plasma samples, via an HPLC system with fluorescence detection.

The synthesis of two side chain functionalised analogues of vitamin K (**3.70** and **3.80**) is presented in chapter three. Two synthetic routes to side chain functionalised vitamin K analogues were investigated. The first involved the conjugation of a functionalised allylic bromide (**3.60**) to a protected naphthoquinone core (**3.11**). The second approach developed involved the functionalisation of the natural isoprene side chain of vitamin K. This method was applied to a K vitamin synthesised via cuprate coupling of **3.11** and geranyl bromide, and to vitamin K<sub>2</sub> (MK-4).

The results of initial immunological studies are reported in chapter four. The production of antibodies recognising the naphthoquinone core of vitamin K was unsuccessful, postulated to be due to the 'bridge effect' leading to antibodies that recognise the isoprenoid side chain of the vitamin K analogue. From the results of these studies three short chain vitamin K analogues (**4.5**, and **4.7**) were designed. Synthesis of **4.5** proceeded via oxidation of an aldehyde intermediate from the

synthesis of **3.70**. Compound **4.7** was obtained from the cuprate coupling of core **3.11** with ethyl bromoacetate, and subsequent functional group interconversions and deprotection. The coupling of analogues **3.70**, **4.5**, and **4.7** to protected lysine and a protected gly-lys dipeptide was investigated to model the coupling of these compounds to a carrier protein for immunological study. The conjugates produced (**4.2**, **4.4**, and **4.13-4.15**) were subsequently studied by NMR spectroscopy and mass spectrometry.

The above synthetic work is applied to the structurally related quinone coenzyme Q and is presented in Chapter five. Using the synthetic methods developed for the synthesis of vitamin K analogues two carboxylic acid functionalised analogues of coenzyme Q (**5.37** and **5.38**) were synthesised. These analogues were also conjugated to protected lysine and gly-lys dipeptide, and studied by NMR spectroscopy and mass spectrometry.

**ABBREVIATIONS**

Å	angstroms
Anal.	microanalysis
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BGP	bone Gla protein
brs	broad singlet
BSA	bovine serum albumin
Calcd.	calculated
CAN	ceric ammonium nitrate
CIGAR	constant time inverse-detected gradient accordion rescaled long-range heteronuclear multiple bond correlation
CoQ	coenzyme Q
δ	chemical shift (NMR)
d	doublet
dd	doublet of doublets
DMAP	<i>N,N</i> -dimethylaminopyridine
DMF	dimethylformamide
DMS	dimethylsulfate
dppp	1,3-bis(diphenylphosphino)propane
dt	doublet of triplets
EDCI	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide. HCl
ELISA	enzyme-linked immunosorbent assay
FTIR	fourier transform infrared spectroscopy
Gla	γ-carboxyglutamic acid
Glu	glutamic acid
HAT	hypoxanthine, aminopterin and thymidine
HDN	haemorrhagic disease of the newborn
HMBC	heteronuclear multiple bond connectivities
HPLC	high-performance liquid chromatography
HPRT	hypoxanthine phosphoribosyl transferase
HRMS	high-resolution mass spectrometry

HRP	horseradish peroxidase
HSQC	heteronuclear single quantum correlation
$\lambda_{\text{em}}$	emission wavelength
$\lambda_{\text{ex}}$	excitation wavelength
$\lambda_{\text{max}}$	maximum wavelength
$J$	coupling constant
LDL	low density lipoprotein
m	multiplet
MCA	monoclonal antibody
MGP	matrix Gla protein
MK-n	menaquinone-n
PAH	polynuclear aromatic hydrocarbon
PTPase	protein-tyrosine phosphatase
q	quartet
rpm	revolutions per minute
RP	reverse phase
s	singlet
t	triplet
TEA	triethylamine
Tg	thyroglobulin
THF	tetrahydrofuran
THP	tetrahydropyranyl
TLC	thin-layer chromatography
VSMC	vascular smooth muscle cell



# **CHAPTER ONE**

## **INTRODUCTION**

## 1.1 VITAMIN K

Vitamins are cofactors, required in minute amounts for the maintenance of growth and many biochemical processes. These organic compounds are not synthesised by the body but are produced by plants and lower animals, and are a dietary requirement for good health. A deficiency of any given vitamin will lead to a disease state. The vitamins, all of which are denoted by letters, may be classified into two groups, the water-soluble vitamins (including vitamins B and C) and the fat-soluble vitamins (including vitamins A, D, E, and K). The role of the vitamins in biochemical systems is extremely diverse, from the growth and repair of body tissues, and the synthesis and degradation of amino acids, to adsorption of minerals such as calcium and phosphorus. A balance of many vitamins is also necessary for the maintenance of healthy teeth, bones and gums, and aids in the digestion of fats and carbohydrates. Each vitamin may function in many diverse ways and in conjunction with a range of other elements.

The K family of vitamins are fat-soluble vitamins, which since their early discovery have been implicated in processes such as blood coagulation, bone metabolism and cell growth. Vitamin K-dependent proteins have been identified in a variety of human tissues including the kidneys, spleen, lungs, uterus, placenta, thyroid, thymus, testes and bones.<sup>1</sup> Some of the conotoxins isolated from the marine snail *Conus* are also thought to be dependent on vitamin K for synthesis.<sup>2</sup> Vitamin K is responsible for the post-translational conversion of glutamic acid residues to  $\gamma$ -carboxyglutamic acid residues in these proteins.

### 1.1.1 HISTORY

Vitamin K was first discovered by Henrik Dam who was studying the possible essential role of cholesterol in the diet of the chick. During his studies Dam noticed that chicks fed on a diet which had been extracted with non-polar solvents had impaired blood clotting.<sup>3</sup> Subsequent studies showed that this condition could not be reversed by the administration of any of the known vitamins or physiologically active lipids.<sup>3</sup> In 1935 Dam proposed that the antihaemorrhagic vitamin of the chick was a new fat-soluble vitamin which he called vitamin K (the first letter of the German word

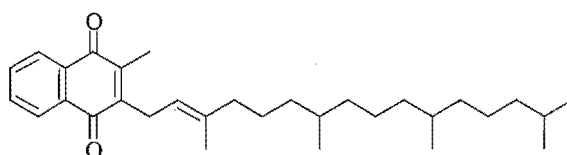


*Koagulation*).<sup>4</sup> The active vitamin was first isolated from alfalfa as a yellow oil and was characterised as 2-methyl-3-phytyl-1,4-naphthoquinone (see figure 1).<sup>3</sup> The identification was confirmed by independent analysis and synthesis of this compound by Almquist and Klose,<sup>5</sup> and Fieser<sup>6</sup> in 1939.

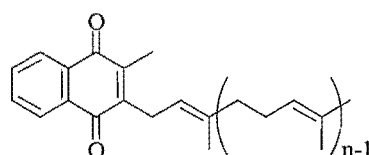
A form of the vitamin was also isolated from putrefied fish meal, but in contrast to the oil isolated from alfalfa this was isolated as a yellow crystalline solid.<sup>3</sup> Studies showed that this compound, called vitamin K<sub>2</sub>, contained an unsaturated side chain at the 3 position of the naphthoquinone core (see figure 1).<sup>3</sup> Early investigations recognized that many sources of this vitamin contained a series of compounds differing in the number of carbons in the polyprenyl side chain.<sup>3</sup> Compounds with 4 – 13 isoprene units are now recognised as being commonly found throughout nature.

### 1.1.2 NOMENCLATURE AND ACTIVITY

The term vitamin K is commonly used to describe any compound that displays vitamin K activity. More specifically the form of the vitamin with a phytyl chain at the 3 position is termed vitamin K<sub>1</sub> or phyloquinone, and the series with repeating unsaturation in the isoprene side chain vitamin K<sub>2</sub>, or the menaquinones. The menaquinones may be denoted MK-n, where n indicates the number of isoprene units in the side chain (see figure 1.1).



Vitamin K<sub>1</sub> - Phylloquinone



Vitamin K<sub>2</sub> - Menaquinone (MK-n)

**Figure 1.1:** Structure of the K vitamins

The other form of vitamin K commonly encountered is 2-methyl-1,4-naphthoquinone, vitamin K<sub>3</sub> or menadione, which lacks any substitution at the 3 position. Although biologically active *in vivo* this compound is not found in nature.

It has, however, been extensively used as a vitamin K supplement, especially in animal feeds.

The stereochemistry at the side chain alkene of vitamin K<sub>1</sub> has been shown to be essential for biological activity.<sup>7</sup> The natural form of vitamin K<sub>1</sub> and the MK's has a *trans* configuration, and the synthetic *cis* isomer exhibits little or no activity.<sup>7,8</sup> Of the menaquinone series MK-3 to MK-5 appear to be the most biologically active *in vivo*.<sup>3</sup> The longer chain menaquinones have been shown to be highly active *in vitro* but they may be less well absorbed, due to increased hydrophobicity, leading to a lower biological activity.<sup>9</sup> The unnatural vitamin K<sub>3</sub> has also been shown to exhibit biological activity but it is unknown if this is alkylated *in vivo* to produce an active compound.<sup>3</sup>

The investigation of the biological activity of a series of analogues of vitamin K has given some structure activity information on these compounds.<sup>3</sup> 2-Methyl-4-amino-1-naphthol and 2-methyl-1-naphthol were shown to have biological activity similar to menadione.<sup>3</sup> The diphosphate, disulfate, diacetate and dibenzoate forms of the reduced K series exhibited full biological activity,<sup>3</sup> possibly due to the fact that it is the reduced form of vitamin K that is biologically active. The methyl substituent at the 2 position is essential for activity, with the 2-ethyl and other alkylated derivatives showing no activity.<sup>7,8</sup> In addition to this the 2-chloro and 2-bromo derivatives are both potent antagonists of vitamin K.<sup>3</sup> 2-Phenyl-1,4-naphthoquinone has been shown to exhibit biological activity, however it is thought to be methylated *in vivo* to vitamin K<sub>1</sub>.<sup>3</sup> Saturation of the double bond of vitamin K<sub>1</sub> reduces activity considerably, but still exhibits more activity than branched chain K<sub>1</sub> analogues.<sup>3</sup> Finally alteration of the naphthoquinone core in any manner also leads to a loss in biological activity.<sup>3</sup>

### 1.1.3 SOURCES OF VITAMIN K

As vitamin K is not synthesised by higher animals it is necessary to obtain the required daily allowance through dietary sources. The K vitamins are synthesised via the shikimic acid pathway, which is also utilised in the production of the aromatic amino acids.<sup>3</sup> The various prenyl side chains are synthesised via the terpene biosynthetic pathway, before incorporation in the final compound.<sup>10</sup>

Vitamin K<sub>1</sub> is synthesised by plants and it is found at significant levels in green leafy vegetables, certain legumes, and some vegetable oils such as rapeseed and

soyabean oil.<sup>11</sup> Most fish, skeletal meats, cereals and beverages also contain low, but measurable amounts of vitamin K<sub>1</sub>.<sup>11</sup>

Vitamin K<sub>2</sub> is predominantly synthesised by anaerobic bacteria including many species that inhabit the lining of the gut. The major forms of vitamin K<sub>2</sub> from this source are MK-10 and MK-11 produced by the *bacteroides*, MK-8 from *enterobacteria*, MK-7 from *veillonella sp* and MK-6 from *Eubacterium lentum*.<sup>11</sup> Vitamin K<sub>2</sub> is also available through the diet in animal livers and fermented foods, including cheeses.<sup>11</sup>

Another potential source of vitamin K is via dihydrovitamin K<sub>1</sub>, which is produced during hydrogenation of vegetable oils, and found in significant quantities in foods prepared using these.<sup>12</sup> Dihydrovitamin K<sub>1</sub> has been detected in human plasma after the consumption of these foods but its bioavailability is unknown.<sup>12</sup> As it is estimated that in America dihydrovitamin K<sub>1</sub> may account for up to 30% of the daily vitamin K intake, this could become very important in assessing vitamin K status.<sup>12</sup>

#### 1.1.4 ABSORPTION, TRANSPORT AND STORAGE

The intestinal absorption of vitamin K is a similar process to that of other fat soluble vitamins and lipid-soluble nutrients. This process, which takes place in the small intestine, involves the solubilization of vitamin K into mixed micelles composed of bile salts and the products of pancreatic lipolysis.<sup>7</sup> Consistent with this model is the observation that patients who lack bile salts or pancreatic enzymes have impaired vitamin K absorption.<sup>7</sup> Once in the mucosal cells of the intestine vitamin K is incorporated into chylomicrons and enters the circulation via the thoracic-duct lymph, chemically unchanged.<sup>7</sup> After hydrolysis by the capillary endothelium, chylomicron remnants are taken up by the liver and a fraction of vitamin K is resecreted into the circulation after incorporation into very low density lipoproteins (VLDL).<sup>7</sup> A healthy adult has been shown to absorb approximately 80% of an oral dose of vitamin K<sub>1</sub>.<sup>7</sup>

Vitamin K is transported between tissues by triglyceride rich lipoproteins,<sup>11</sup> which accounts for the fact that patients with hyperlipidaemia show grossly elevated plasma levels of vitamin K.<sup>13</sup> This is also consistent with the finding that plasma vitamin K<sub>1</sub> levels increase with age as do the corresponding plasma triglyceride

concentrations.<sup>14</sup> The main storage site for vitamin K is the liver, but it is also found in many other tissues including the adrenal glands, lungs, bone marrow, kidneys, and lymph nodes.<sup>3</sup> The distribution of the different vitamers of K varies between tissues with vitamin K<sub>1</sub> the predominant form in the heart and liver and relatively high amounts of MK-4 detected in the brain, kidney and pancreas.<sup>15</sup> The accumulation of vitamin K in the heart is of particular interest as no vitamin K-dependent carboxylase activity has been observed in this tissue.<sup>16</sup> This may mean that there is another function of vitamin K as yet undetermined. The detection of MK-4 in almost all tissues is also of interest, as MK-4 is not found in significant amounts in any food sources.<sup>15</sup> Dietary vitamin K<sub>1</sub> has been shown to be a source of tissue MK-4, suggesting some unknown cellular role for this particular vitamer.<sup>15</sup>

Although vitamin K is stored in a variety of tissues the half life is thought to be short, with the entire body pool of vitamin K being replaced as often as every two and a half hours.<sup>3</sup>

The bioavailability of vitamin K from various sources is still under debate. Although green leafy vegetables are high in phylloquinone, absorption of the vitamin through the intestine is low,<sup>14</sup> as the vitamin is tightly bound to the thylakoid membrane of the chloroplasts.<sup>17</sup> The absorption of menaquinones produced in the gut is also uncertain. The detection of significant amounts of MK-10 to MK-13 in human liver, with no significant levels detected in foods, would suggest that these compounds originate from intestinal synthesis.<sup>18</sup> Conversely it has been suggested that absorption of the highly lipophilic menaquinones is unlikely as they are tightly bound to the cytoplasmic membrane and in the presence of bile salts the only perceivable mechanism for absorption would be via the portal route.<sup>19</sup>

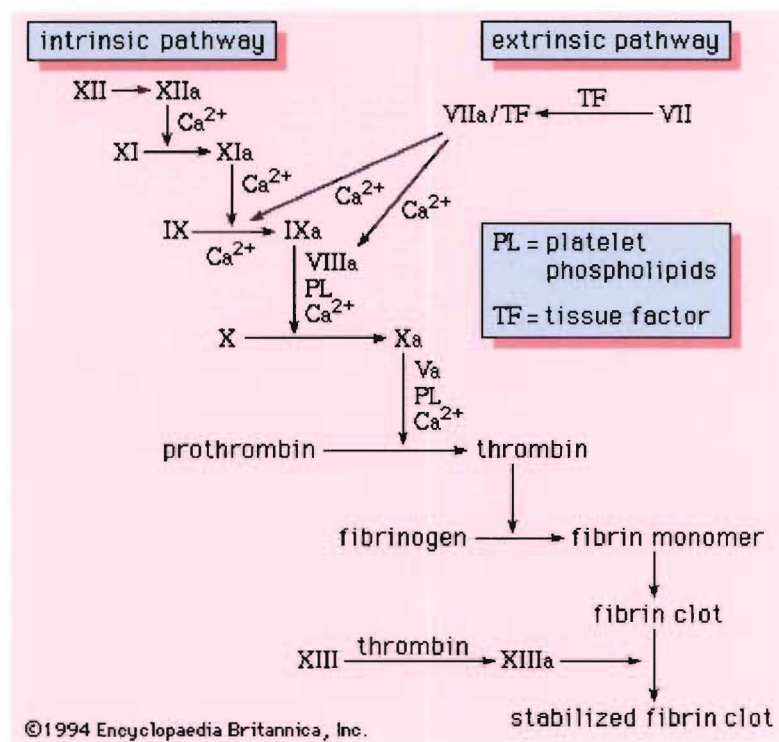
Having been absorbed through the intestine, and transported to sites of action or storage, vitamin K will act in a number of different biochemical roles.

### 1.1.5 VITAMIN K AND BLOOD CLOTTING

As implied by the experiments of Dam<sup>4</sup> which led to the discovery of vitamin K, this family of compounds is important in blood clotting. In the mid 1950's, and the following decade, clotting factors VII, IX, X and prothrombin were discovered as being vital to blood clotting, and dependant on vitamin K for synthesis.<sup>3</sup> The plasma glycoproteins, or clotting factors, involved in blood coagulation are synthesised as

proenzymes, and activated as required in order to control the clotting process. These proteins, including factors XII, XI, IX, VII and prothrombin, are zymogens of serine proteases.<sup>20</sup>

Blood clotting is a complicated biochemical process that consists of 2 major pathways, the intrinsic pathway where all the protein components are contained within the blood, and the extrinsic pathway where the cell membrane tissues play an important role (see figure 1.2).<sup>20</sup> The intrinsic pathway begins with the activation of factor XII by proteins, including kininogen and kallikrein.<sup>21</sup> This is initiated by damage to non-endothelial cells which leads to activation of prekallikrein to kallikrein, in turn activating factor XII to factor XIIa (activated factor XII).<sup>22</sup> When activated each protein in turn acts to cleave the next protein in the cascade, often in conjunction with other factors or bound calcium ions, and in this way the intrinsic pathway involves the activation of factors XII, XI and IX.<sup>22</sup> The activation of factor X to factor Xa involves not only factor IXa, but also factor VIIIa which is not a serine protease, but is thought to act as a receptor for factor IXa.<sup>23</sup>



**Figure 1.2:** The blood clotting cascade

The extrinsic pathway is a rapid response mechanism to tissue injury via the production of activated tissue factor (TF).<sup>23</sup> Tissue factor activates factor VII to factor VIIa, which is then able to activate factor X to Xa.<sup>23</sup> At this point the extrinsic

and intrinsic pathways converge. Factor Xa is a serine protease which binds to the phospholipid membranes released by platelet aggregation, and cleaves prothrombin to thrombin.<sup>21</sup> The activation of prothrombin occurs on the platelet and requires calcium ion mediated binding of prothrombin to the anionic phospholipid head groups via vitamin K-dependent  $\gamma$ -carboxyglutamic acid residues. The platelets also contain factor V which, when activated to Va, acts as a receptor for factor Xa.<sup>23</sup> Cleavage of prothrombin, and subsequent release of thrombin leads to clot formation by activation of fibrinogen to fibrin, the structural protein that assembles into the fibrin polymer of the clot.<sup>20</sup>

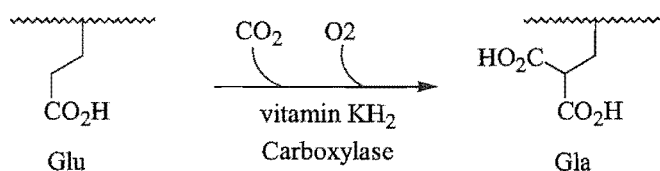
The blood-clotting cascade is tightly controlled, and this process involves three vitamin K-dependent anticoagulation proteins, protein C, protein S and protein Z. Although protein Z has been described its function is unknown.<sup>3</sup> Protein C is a zymogen of a serine protease which is converted to activated protein C by thrombin, and inactivates factors Va and VIIa by limited proteolysis thereby halting coagulation.<sup>2</sup> Thrombin, upon binding to the endothelial cell receptor thrombomodulin changes conformation allowing the activation of protein C, and in turn converting thrombin from a strong procoagulant to an anticoagulant.<sup>22</sup> The presence of thrombomodulin on healthy endothelial cells to bind any circulating thrombin prevents the formation of clots in undamaged vessels.<sup>22</sup>

Protein S acts as a non-enzymatic cofactor for activated protein C. The exact mechanism by which this occurs is unknown, but a role in anticoagulation is suggested, as patients deficient in protein S display problems associated with the control of the clotting process.<sup>2</sup> Approximately 50% of circulating protein S is bound as a bimolecular complex to C4b-binding protein and it has been shown that only free protein S is active as a cofactor.<sup>2,17</sup>

Although vitamin K is involved in these two opposing pathways the mechanism by which it is involved in post-translational modification of the proteins is the same.

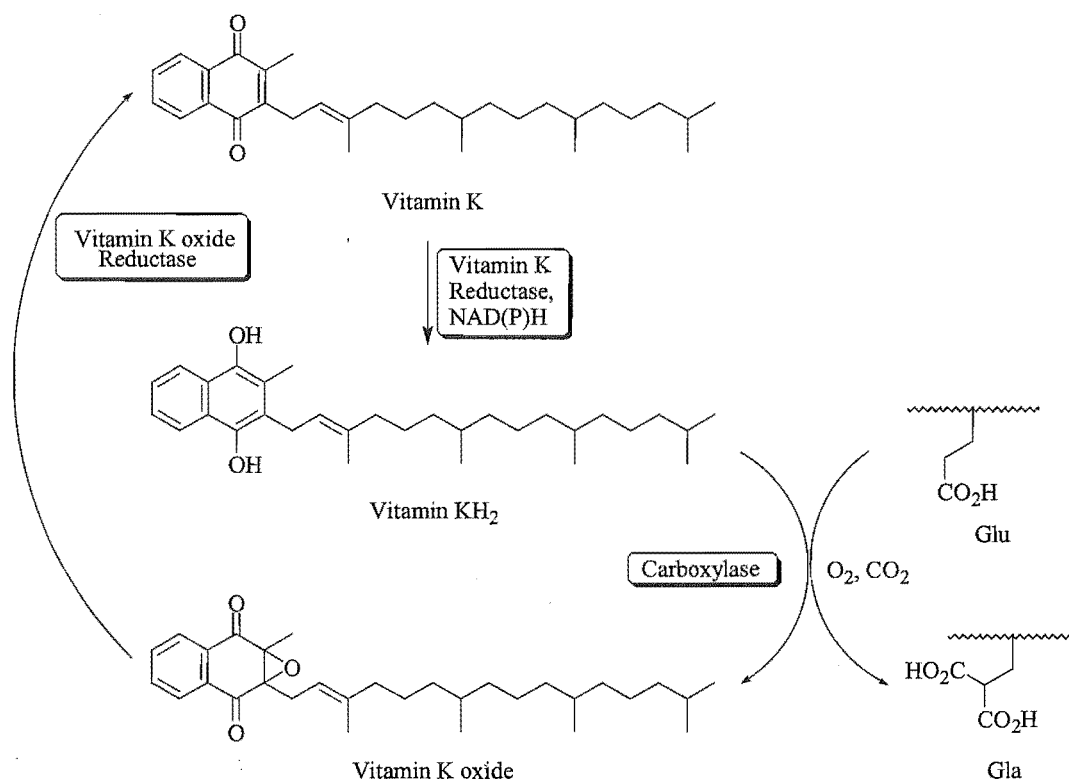
### 1.1.6 VITAMIN K CYCLE

Vitamin K in the presence of vitamin K carboxylase, molecular oxygen and carbon dioxide will selectively carboxylate specific glutamic acid (Glu) residues to  $\gamma$ -carboxyglutamic acid (Gla) residues in a protein precursor (see figure 1.3).<sup>11</sup>



**Figure 1.3:** Conversion of Glu to Gla by vitamin K –dependent carboxylase

The process shown in figure three is the product of a catalytic cycle involving vitamin K, sometimes referred to as the vitamin K cycle (see figure 1.4). The active form of vitamin K *in vivo* is the hydroquinone (KH<sub>2</sub>), produced from vitamin K by a nicotinamide dinucleotide phosphate (NAD(P)H) dependent flavoprotein reductase.<sup>24</sup> During the course of the carboxylation reaction Glu is converted to Gla and vitamin KH<sub>2</sub> is converted to vitamin K oxide.<sup>24</sup> As any carboxylation event will be energetically unfavourable, it must be linked to an energetically favourable process to generate a product. In this case the energy is provided by the direct oxidation of vitamin KH<sub>2</sub> to vitamin K oxide.<sup>2</sup> Whether the carboxylation and oxidation are concerted and require only one enzyme, or involve 2 separate enzymes is still largely unresolved with literature supporting both hypotheses.<sup>24</sup> The final step of the cycle is the reduction of vitamin K oxide to vitamin K by vitamin K oxide reductase.<sup>24</sup>



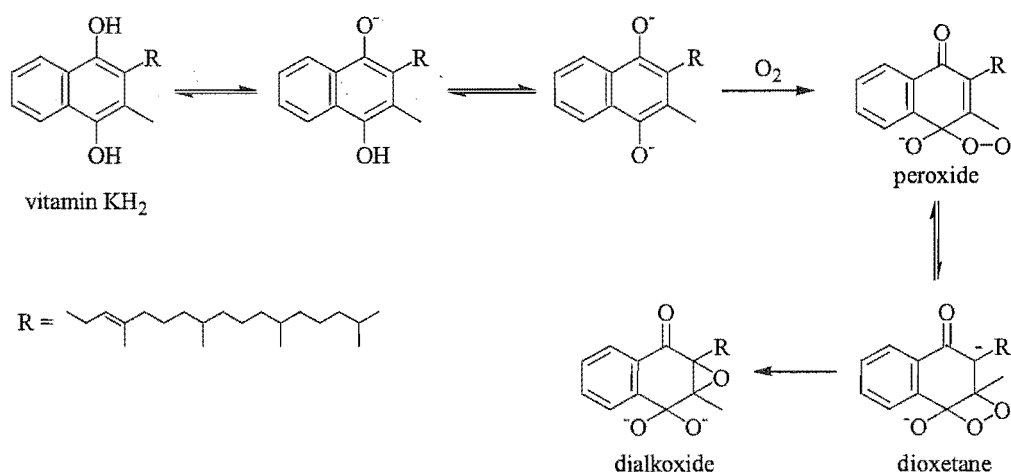
**Figure 1.4:** The vitamin K cycle

The anticoagulant drugs warfarin and dicumarol act by inhibiting vitamin K reductase and vitamin K oxide reductase.<sup>25</sup> This interrupts the catalytic cycle of vitamin K and therefore ceases the production of the vitamin K-dependent clotting factors.<sup>7</sup> Although vitamin K can no longer be recycled, and builds up in the liver as vitamin K oxide, dietary vitamin K can still feed into the cycle, through reduction by a second NAD(P)H dependent enzyme which is insensitive to warfarin.<sup>7</sup> For this reason anticoagulant therapy is dependent on a balance between the inhibition of the recycling enzymes and the amount of dietary vitamin K which can enter the cycle.<sup>7</sup> Certain antibiotics have also been shown to inhibit vitamin K oxide reductase, namely those bearing a *N*-methylthiotetrazole (NMTT) side chain (e.g. cefamanadole) or a methylthiadiazole-thiol (MTD) side chain (e.g. cefazoin).<sup>9</sup>

Although the vitamin K cycle has been established for some time the exact mechanism by which vitamin K effects the carboxylation of Glu to Gla in the presence of vitamin K-dependent carboxylase is still a matter of some debate. The carboxylation of Glu to Gla requires the cleavage of a CH bond and replacement with a carboxyl group. It has been proposed that this occurs via a free-radical or anionic intermediate.<sup>24</sup> In 1991 Paul Dowd *et al.* proposed a novel base strength



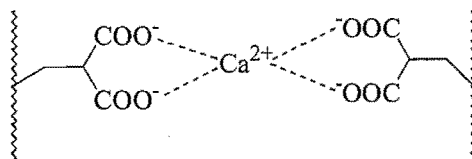
amplification mechanism for this carboxylation reaction (see figure 1.5).<sup>24</sup> This hypothesis was established in an attempt to explain how vitamin K could be transformed into a base strong enough to remove a proton from Glu. Vitamin  $\text{KH}_2$  is moderately acidic and can therefore ionize in the enzymatic environment to produce equilibrium concentrations of the mono- and dianion.<sup>25</sup> Reaction of the dianion with oxygen will yield a peroxide anion intermediate which can add to the enone to give a dioxetane. This can then further rearrange by ring opening to give the dialkoxide.<sup>25</sup> It is proposed that this dialkoxide could remove the proton from Glu as required for carboxylation.<sup>25</sup> A similar series of reaction and rearrangements from the monoanion of vitamin  $\text{KH}_2$  would produce a tertiary alkoxide also capable of proton abstraction.<sup>24</sup> Dowd *et al.* have carried out a series of model experiments<sup>24</sup> to substantiate this hypothesis and at present this is the generally accepted carboxylation mechanism.



**Figure 1.5:** Proposed mechanism of action of vitamin K

### 1.1.7 GLA FORMATION AND PROTEIN ACTIVATION

Upon this post-translational modification of Glu residues the protein is able to chelate calcium ions in the geminal carboxylate groups of the newly formed Gla residues. In the instance of blood coagulation, the most studied of processes involving Gla containing proteins, this calcium ion may then form ion bridges to the phosphate head groups at phospholipid membrane surfaces of blood platelets and endothelial cells.<sup>25</sup> Introduction of the Gla carboxyl group is also believed to confer structural changes upon the protein by calcium ion mediated intrachain Gla-Gla interactions (see figure 1.6).<sup>25</sup>



**Figure 1.6:** Interaction of Gla residues with a calcium ion

In most Gla proteins identified, including all the K-dependent clotting factors, all the Gla residues fall within a short sequence at the amino terminal of the peptide. This area is termed the Gla domain, and with the solving of the X-ray crystal structure of the Gla domains of several clotting factors more information on the role of Gla in the activation of these proteins has become available.<sup>2</sup> It was found that the Gla residues point inwards towards calcium ions bound in the interior of the Gla domain. Studies have also shown that Gla itself has a poor affinity for calcium ions, and groups of Gla residues do not create functional calcium binding sites without correct folding of the parent protein.<sup>2</sup>

The fact that vitamin K-dependent carboxylase could effect the carboxylation of various numbers of Glu residues in different proteins without affecting the rest of the protein raised several questions about the specificity and recognition of the carboxylase for the vitamin K-dependent protein. Identification of the cDNA sequences for the known vitamin K-dependent proteins revealed an homologous peptide that is the recognition sequence for the carboxylase.<sup>26</sup> In most cases this is a propeptide which is cleaved before the release of the peptide into circulation.<sup>26,27</sup> Although the level of carboxylation varies between proteins, a certain amount of homology has also been observed in the Gla domain of most of the known vitamin K-dependent proteins.<sup>26</sup> The identification of these recognition sequences has lead to the identification of previously unknown vitamin K-dependent proteins in various body tissues.

### 1.1.8 VITAMIN K AND HDN

A clinical vitamin K deficiency is rarely observed in adults, unless excessive antibiotic treatment or other medical conditions (e.g. liver complaints) accompany this diagnosis. However, in neonates, spontaneous bleeding due to vitamin K deficiency is a well-known risk.<sup>11</sup> This condition termed haemorrhagic disease of the newborn

(HDN), or vitamin K deficient bleeding, was first described in 1894, with a treatment of fresh cows milk being prescribed.<sup>1</sup> All neonates are born deficient in vitamin K, but several other factors have been identified as increasing the risk of HDN.

There are several factors contributing to the low vitamin K status of newborns. It has been shown that vitamin K is not readily transferred across the placenta,<sup>1,28</sup> this could be partly due to the low lipid concentrations in cord plasma.<sup>13</sup> Hepatic stores of menaquinones are also known to be virtually non existent in neonates.<sup>11</sup> The administration of anticonvulsant drugs during pregnancy, e.g. warfarin, or a stressful pregnancy and delivery have been shown to increase the risk of HDN.<sup>29</sup>

HDN may be classified into three syndromes: early HDN when bleeding occurs within 24 hours of birth, classic HDN when bleeding occurs on days one to seven, and late HDN when bleeding occurs after the first week of life.<sup>32</sup> Bleeding in early HDN is often intracranial, intrathoracic or intraabdominal, and maternal drugs are a frequent cause.<sup>11</sup> In the case of classic HDN bleeding is often gastrointestinal, under the skin or nasal and this is mostly an idiopathic disorder.<sup>11</sup> Late onset HDN is often identified by intracranial bleeding which may be fatal or cause morbidity, this is also often idiopathic and may be the presenting feature of underlying disease eg cystic fibrosis<sup>11,32</sup> or subclinical liver dysfunction.<sup>33</sup> Late onset HDN is almost entirely confined to exclusively breast-fed infants who did not receive vitamin K prophylaxis at birth.<sup>28,34</sup>

Estimating the incidence of HDN in an unprotected population is difficult, and there is large intercountry variation, with estimates of incidence of late HDN (per 100000 births) of 4.4 in UK, 7.2 in Germany, 10.5 in Japan, and 72 in Thailand.<sup>11</sup> The incidence of bleeding due to vitamin K deficiency has been shown to be 15-20 fold higher in solely breast-fed infants.<sup>30</sup> Purely breast-fed infants are at a higher risk for two reasons. Firstly vitamin K levels of breast-milk have been shown to be a lot lower than levels in cows milk and infant formulas (which are fortified with vitamin K).<sup>31</sup> Also the bacteria in breast milk which go on to colonise the intestine of the infant are unable to produce menaquinones, unlike the organisms often associated with intestinal flora of formula fed infants.<sup>1</sup>

Since the observation that HDN is responsive to vitamin K, the generally accepted practise has been vitamin K prophylaxis at birth.<sup>35</sup> The two main routes of administration of vitamin K are oral (by three consecutive doses) or intramuscular (via a single injection at birth). There has long been much debate about the most

effective prophylactic treatment, as well as many investigations into the possibility of administering vitamin K to breast-feeding mothers.<sup>36</sup> The debate over vitamin K prophylaxis was again raised in 1992 when Golding *et al* published the results of a study which suggested a possible link between intramuscular, but not oral, vitamin K prophylaxis, and an increased incidence of childhood cancer.<sup>37</sup> There were two possible reasons for this put forward. Firstly very high levels of vitamin K (5000 times normal) have been shown to increase sister chromatid exchanges in human placental lymphocytes *in vitro*.<sup>37</sup> The site of intramuscular injection is thought to act as a store and slowly release the vitamin into circulation possibly setting up this increased risk of sister chromatid exchange, however one small study involving six infants showed no evidence of this occurring *in vivo*.<sup>37</sup> The second hypothesis is that it isn't the vitamin K itself but other ingredients in the preparation that are carcinogenic.<sup>37</sup> Phenol and vitamin K may provide reactions needed for carcinogenesis, and vitamin K has been shown to increase the mutagenicity and carcinogenicity of benzopyrene also found in some K preparations.<sup>37</sup> Alternatively Golding postulates that rather than the vitamin K being harmful a deficiency state may be protective, and protect vulnerable tissue from mutagenesis during this critical stage of rapid growth and development.<sup>37</sup> Since this study several other groups have tried to confirm or discount the link suggested by Golding. These results are still inconclusive. A study in the United States showed no link between intramuscular vitamin K and cancer<sup>38,39</sup> and more recently four British studies have failed to resolve this debate. Two of these studies suggested a increased risk of cancer with intramuscular K prophylaxis, although with a risk factor much smaller than that reported by Golding, and the remaining two studies found no link between the points in question.

With no risk shown by any group between cancer and oral vitamin K prophylaxis this became the preferred route of administration for some time. However, independent studies have shown that oral prophylaxis is not as effective against classic and late HDN, especially if all three doses are not administered.<sup>34</sup> The possibility of targeting breast-fed infants by giving vitamin K supplements to breast-feeding mothers has also been investigated. Supplementation of vitamin K has been shown to dramatically increase breast milk and plasma concentrations in the mother, but an increased dietary intake of vitamin K showed no appreciable increase in vitamin K content in breast milk.<sup>35</sup> However, no significant correlation between

plasma phylloquinone concentration of the infant and breast milk phylloquinone concentration was observed, implying that absorption of the K vitamins may be the problem.<sup>35</sup> It was also shown that when all infants were given prophylaxis at birth no difference in the vitamin K-dependent clotting factors could be demonstrated between formula and breast-fed infants, implying almost full protection from HDN.<sup>35</sup>

### 1.1.9 VITAMIN K AND BONE HEALTH

Following the study of the vitamin K-dependent clotting factors, Gla containing proteins were isolated from bone tissue. Three Gla containing proteins have been isolated in bone to date: osteocalcin (or Bone Gla protein, BGP), matrix Gla protein (MGP) and protein S. It has been demonstrated that adequate vitamin K status is important both for early skeletal development and the maintenance of healthy mature bone.<sup>11</sup> Vitamin K antagonists have been shown to be toxic to bone, and the optimum vitamin K concentration required to maintain function in bone may be higher than that necessary for coagulation.<sup>11</sup> Oral anticoagulants inhibit carboxylation of Gla proteins which vitamin K administration reverses in the liver, but this process is not reversed in the bone.<sup>17</sup> Bone tissue is also a repository for vitamin K and may contain levels as high as the liver.<sup>18</sup> Vitamin K<sub>1</sub> is the major form in bone tissue but MK-4, 5, 6 and 7 are also observed in bone lipid, with concentration decreasing with increasing side chain length.<sup>19</sup>

The most abundant of the vitamin K-dependent bone proteins is osteocalcin, which accounts for up to 15% of the non-collagenous bone.<sup>19</sup> Osteocalcin is a protein of 49-50 amino acid residues including 3 Gla residues at positions 17, 21, and 24,<sup>19</sup> which are essential for calcium binding.<sup>40</sup> The spacing of the Gla residues is consistent with the spacing of calcium ions in hydroxyapatite to which osteocalcin binds tightly.<sup>11</sup> Like all bone Gla proteins osteocalcin is synthesised by osteoblasts, but unlike the other Gla containing bone proteins it is also synthesised by odontoblasts.<sup>17</sup> The precise mechanism of action of osteocalcin is unknown but it functions as a negative regulator of bone formation.<sup>40</sup> A group of osteocalcin-deficient mice were shown to be normal at birth, but by six months of age had developed long bones of increased thickness and density compared to wild-type mice.<sup>40</sup> Undercarboxylated osteocalcin has been shown to be very sensitive to low vitamin K intake,<sup>17</sup> and is now considered a more sensitive marker of vitamin K status

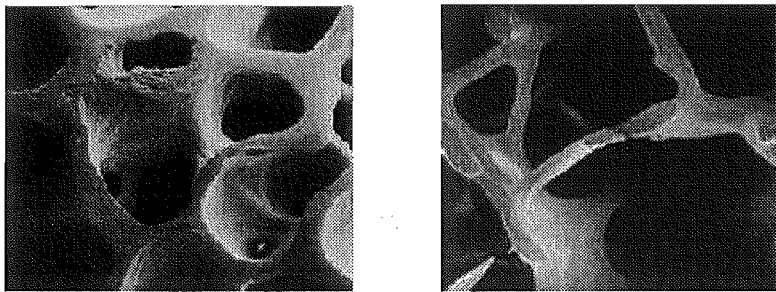
than undercarboxylated clotting factors. Circulating undercarboxylated osteocalcin has been observed to increase with age, especially in post-menopausal women. These levels are also increased in patients with hip-fracture and may be used as an indicator of fracture risk.<sup>19,40</sup> These levels of undercarboxylated osteocalcin respond positively to vitamin K supplementation.<sup>16</sup>

Matrix Gla protein (MGP) is a 9.6 kDa protein which despite its high proportion of hydrophilic amino acids is virtually insoluble in aqueous solutions.<sup>17</sup> Most secreted MGP enters circulation, but it is also abundant in bone and cartilage.<sup>17</sup> MGP has been identified to take part earlier in the calcification process than osteocalcin and binds to both the organic and mineral components of the bone.<sup>40</sup> MGP inhibits calcification of arteries and cartilage through its mineral-ion-binding ability,<sup>40</sup> and mice without the genes necessary for MGP synthesis displayed increased heart rate, decreased stature, osteopenia, fractures and eventually died due to calcification of the aorta.<sup>40</sup> However, no atherosclerotic plaques were present in these animals, implying the formation of such plaques is controlled by a gene independent of the MGP genes, but MGP may affect calcification once plaques are formed.<sup>40</sup> The role of MGP in inhibition of calcification has been proposed as a mechanism for maintaining the organization of chondrocytes necessary for longitudinal bone growth.<sup>40</sup> MGP is not exclusive to bone tissue, it is also found in cartilage and expressed in a variety of other soft tissues.<sup>19</sup> The teratogenic effect of warfarin in causing excessive fetal calcification is at least partly due to the inhibition of MGP function by the vitamin K antagonist.<sup>19</sup>

The final vitamin K-dependent protein isolated from bone tissue is protein S, which has already been discussed as an anticoagulant in the blood clotting cascade.<sup>19</sup> Protein S is a single chain molecule containing 11 Gla residues, and is synthesised not only by osteoblasts but also by hepatocytes, megakaryocytes and endothelial cells.<sup>17</sup> The study of two patients with well established protein S deficiency showed severe osteopenia and reduced bone mineral density.<sup>17</sup> This finding, coupled with isolation of protein S from organic bone matrix, suggests that protein S may have an additional role in bone metabolism.<sup>17</sup> The exact nature of this role and its mechanism of action are still unclear.

With evidence of vitamin K-dependent proteins being vital to bone metabolism, and the indication of poor vitamin K status in many fracture patients, the possibility of a link between vitamin K and osteoporosis has been suggested.<sup>41</sup> Osteoporosis is a

metabolic bone disease characterised by a defect in bone remodelling, and loss of normally mineralised bone (see figure 1.7).<sup>41</sup> Bone remodelling is the result of the opposing actions of two cell types in bone.<sup>41</sup> The osteoclasts, attracted to an area of damage, will remove a finite amount of bone before undergoing apoptosis.<sup>41</sup> Osteoblasts then synthesise new bone to replace that which has been removed.<sup>41</sup> Osteoporosis results from an imbalance in this process where osteoblasts fail to replace removed bone leading to lower bone mineral density.<sup>41</sup> There is still debate over any role for vitamin K in osteoporosis, as healthy mature bone appears resistant to the effects of vitamin K antagonists.<sup>42</sup>



**Figure 1.7:** Healthy bone (left) compared with osteoporotic bone (right)\*

Although epidemiological studies are limited, there is support for the role of vitamin K in the retardation of bone loss in the elderly,<sup>41</sup> and in Japan MK-4 is often given at 45mg/day for treatment of post-menopausal osteoporosis.<sup>16</sup> Due to its role in tissue mineralisation, it has been proposed that poor vitamin K status may be the common denominator in osteoporosis and atherosclerosis.<sup>17</sup>

#### 1.1.10 VITAMIN K AND CELL GROWTH

In the search for proteins containing Gla residues, the protein encoded by growth-arrest-specific gene 6 (*gas6*) was identified as the first Gla containing receptor ligand that regulates cell activity.<sup>43</sup> This protein (Gas6) is released from growth-arrested vascular smooth muscle cells (VSMCs) and potentiates VSMC proliferation induced by calcium ion mobilising growth factors.<sup>43</sup> Gas6 shows sequence similarity with protein S, and is a ligand for the receptor tyrosine kinases Axl and Sky.<sup>43</sup> The

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\* Image sourced from [pepper.uchc.edu/bone.jpg](http://pepper.uchc.edu/bone.jpg)

interaction of Gas6 with its receptor requires calcium ion binding and Gla deficient Gas6 shows poor receptor binding and growth potentiating activity.<sup>43</sup> The Gla domain of Gas6 is thought to act as a regulatory domain fixing the protein in an inactive conformation when uncarboxylated, and activating receptor binding when carboxylation occurs.<sup>43</sup> The role of Gas6 in the activity of VSMCs *in vitro* suggest that it contributes to the development and maintenance of the vascular wall, as well as the process of vascular disease.<sup>43</sup>

As well as its role in cell proliferation vitamin K has been implied in the inhibition of growth of several tumour cell lines.<sup>44</sup> As early as 1980 it was recognised that vitamin K<sub>3</sub> inhibited the binding of epidermal growth factor to the membrane receptors.<sup>44</sup> Vitamin K<sub>3</sub> was also found to inhibit the growth of mouse neuroblastoma, rat glioma and mouse melanoma cells at micromolar concentrations.<sup>44</sup> Following this, vitamin K<sub>3</sub> was shown to have similar properties against a series of animal and human tumour cells *in vitro* and *in vivo*, including several drug resistant cell lines.<sup>45</sup> Vitamin K<sub>3</sub> was suggested as a possible agent in combination chemotherapy, and preliminary studies showed that the concentrations required to suppress tumour growth were readily achievable.<sup>45</sup> The mechanism by which vitamin K<sub>3</sub> arrests tumour growth is thought to revolve around the quinone moiety. The quinone can undergo one electron reduction resulting in the formation of a semiquinone, which in turn may react with molecular oxygen to regenerate the quinone and superoxide, similar to that of known anticancer agents diaziquone, mitomycin, and doxorubicin.<sup>45</sup> Vitamin K<sub>3</sub> has been shown to be a potent inducer of ssDNA damage in the absence of topoisomerase, which is believed to play a role in the mechanisms of other quinone anticancer agents.<sup>45</sup> Other studies have suggested that sulfhydryl arylation may be responsible for cell growth inhibitory effects of vitamin K<sub>3</sub>.<sup>46</sup>

Following these studies the investigation of several vitamin K analogues found that 2-(2-mercaptoethanol)-3-methyl-1,4-naphthoquinone (Cpd 5) was a more potent cell growth inhibitor than previously known K vitamins.<sup>46</sup> It was predicted that Cpd 5 was active due to arylation of cellular thiols or thiol dependent proteins, by an addition-elimination mechanism.<sup>46</sup> It was found that Cpd 5 targeted protein-tyrosine phosphatases (PTPases), which play a pivotal role in many cellular functions.<sup>46</sup> Sulfhydryl arylation of a critical cysteine in the active site of PTPases, and subsequent perturbation of protein tyrosine phosphorylation is the suggested mechanism of cell



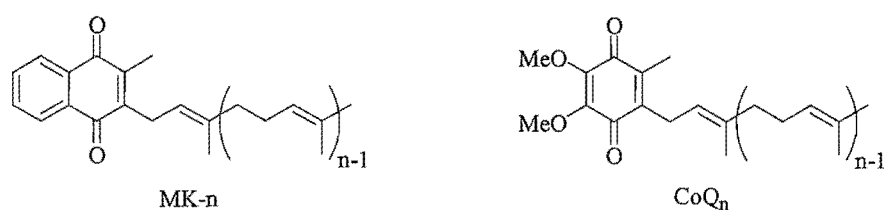
growth inhibition of Cpd 5.<sup>46</sup>

## 1.2 COENZYME Q

Like vitamins, coenzymes are non-protein substances required by proteins for biological activity, especially in enzyme catalysed reactions. These compounds are not consumed in the course of the reaction, but are found unchanged upon its completion. Coenzymes include substances such as nicotinamide-adenine dinucleotide (NAD), flavin-adenine dinucleotide (FAD), pyridoxal phosphate and coenzymes A and Q. Unlike vitamins however, coenzymes are produced by the body and are therefore not a dietary requirement for cellular function.

Coenzyme Q (CoQ), or ubiquinone, is found throughout the body in cell membranes, especially the mitochondrial membranes of the heart, liver, lungs, kidneys, spleen, pancreas and adrenal glands. The total body pool of CoQ is estimated to be 500 mg to 1500 mg.<sup>47</sup> A deficiency of this cofactor has been associated with many varied disease states including heart disease, ageing, cancer, diabetes, stroke, Alzheimer's disease, Parkinson's disease, muscular dystrophy, atherosclerosis and AIDS. CoQ has been found to have two main biochemical functions: as a cofactor in the electron transport chain of the mitochondrial membrane, and as an antioxidant.

The chemical structure of coenzyme Q is closely related to that of the menaquinones (see figure 1.8). The 2-methyl-3-polyprenyl-5,6-dimethoxybenzoquinone exists with varying numbers of prenyl units, with CoQ<sub>10</sub> (containing 10 prenyl units) being the active form in humans.



**Figure 1.8:** Chemical structure of menaquinone-n (MK-n) and coenzyme Q-n (CoQ<sub>n</sub>)

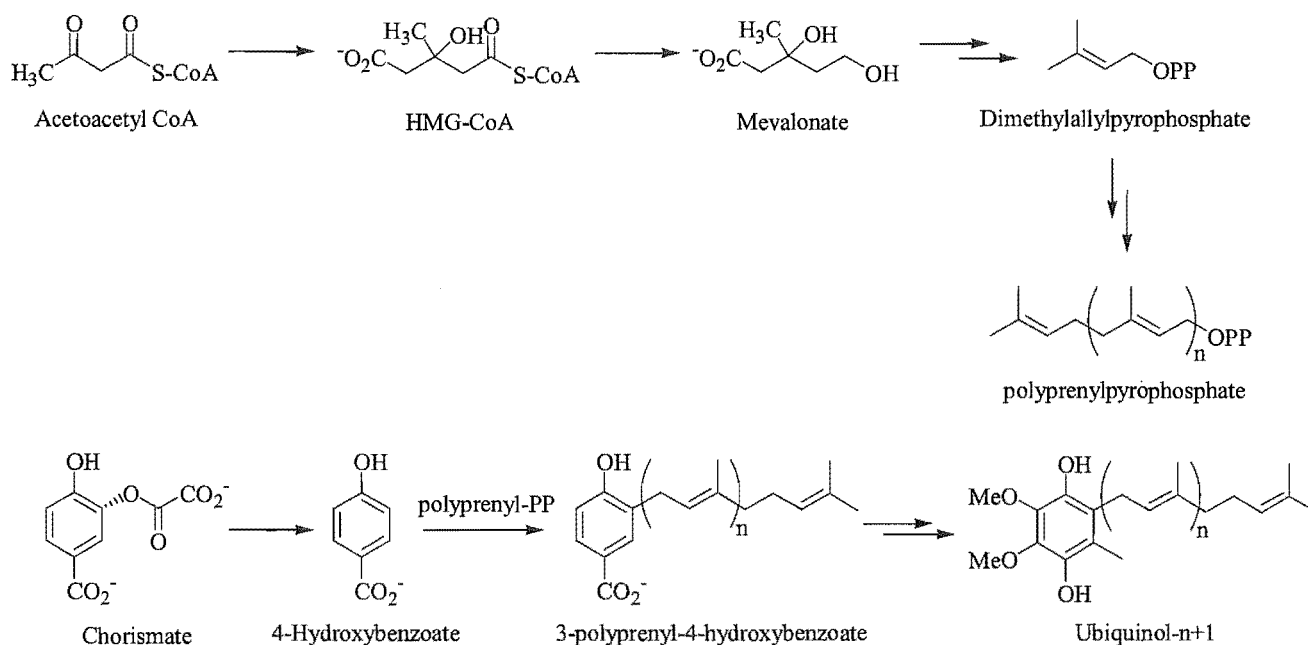
### 1.2.1 HISTORY

Coenzyme Q 10 was first isolated from beef heart mitochondria in 1957 by Dr Fred Crane,<sup>48</sup> and the structure was elucidated by Dr Karl Folkers and reported the following year.<sup>49</sup> It was soon determined that CoQ was found in virtually every cell in the body, but its biological function was still unknown.<sup>50</sup> The role of CoQ in the energy transfer process in mitochondria was elucidated by Dr Peter Mitchell, who was awarded the 1978 Nobel Prize in chemistry for his work in this area.<sup>51</sup>

Coenzyme Q first went into clinical trials in patients with congestive heart disease in 1965 in Japan, and the first documentation of a CoQ deficiency in heart disease patients was published by Littarru and Folkers in 1972.<sup>52</sup> At approximately the same time a CoQ<sub>10</sub> deficiency was also observed in patients with periodontal disease. Clinical trials at this time were difficult due to limited supply of CoQ, but with the introduction of a viable commercial fermentation technique to produce CoQ<sub>10</sub> in significant quantities, in the mid 1970's the number and size of clinical trials for various different human diseases increased dramatically. Although marketed as a drug in Japan, coenzyme Q was marketed as a health supplement in the US in order to hasten its appearance on the market by removing the need for FDA approval. By the 1990's CoQ was widely prescribed in Japan for treatment of heart failure, cardiac arrhythmias and hypertension, and had become popular on the health food market worldwide.

### 1.2.2 BIOSYNTHESIS

The biosynthesis of CoQ takes place in all body tissues, and proceeds from chorismate and a polyprenylpyrophosphate. It is a multistep process which requires at least 8 vitamins and several trace elements.<sup>53</sup> The biosynthesis occurs in three sections; the synthesis of the polyprenyl side-chain via the mevalonate pathway in the microsome, the synthesis of the precursor ring, chorismate, via the shikimate pathway, and the condensation of the two and subsequent functionalisation of the ring which takes place in the Golgi system (see figure 1.9).<sup>54</sup>



**Figure 1.9:** Simplified scheme of ubiquinol biosynthesis

The mevalonate pathway is present in all tissues of higher organisms, and the products of this pathway are cholesterol, CoQ, and the cytoplasmic intermediates farnesyl pyrophosphate and geranylgeranyl pyrophosphate which are used for the isoprenylation of proteins.<sup>54</sup> Geranyl pyrophosphate, which is synthesised in the cytosol, is the substrate for an enzyme at the cytosolic surface of the endoplasmic reticulum (ER) membranes which synthesise solanesyl pyrophosphate.<sup>54</sup> This is then transferred to the lumen of the ER and transported to the Golgi system, but it is not clear in which part of the Golgi system the polyisoprene is utilised.<sup>54</sup> Condensation with the precursor ring occurs at the inner membrane of the Golgi system, followed by functionalisation of the ring. The final product appears at the cytoplasmic surface of these membranes.<sup>54</sup>

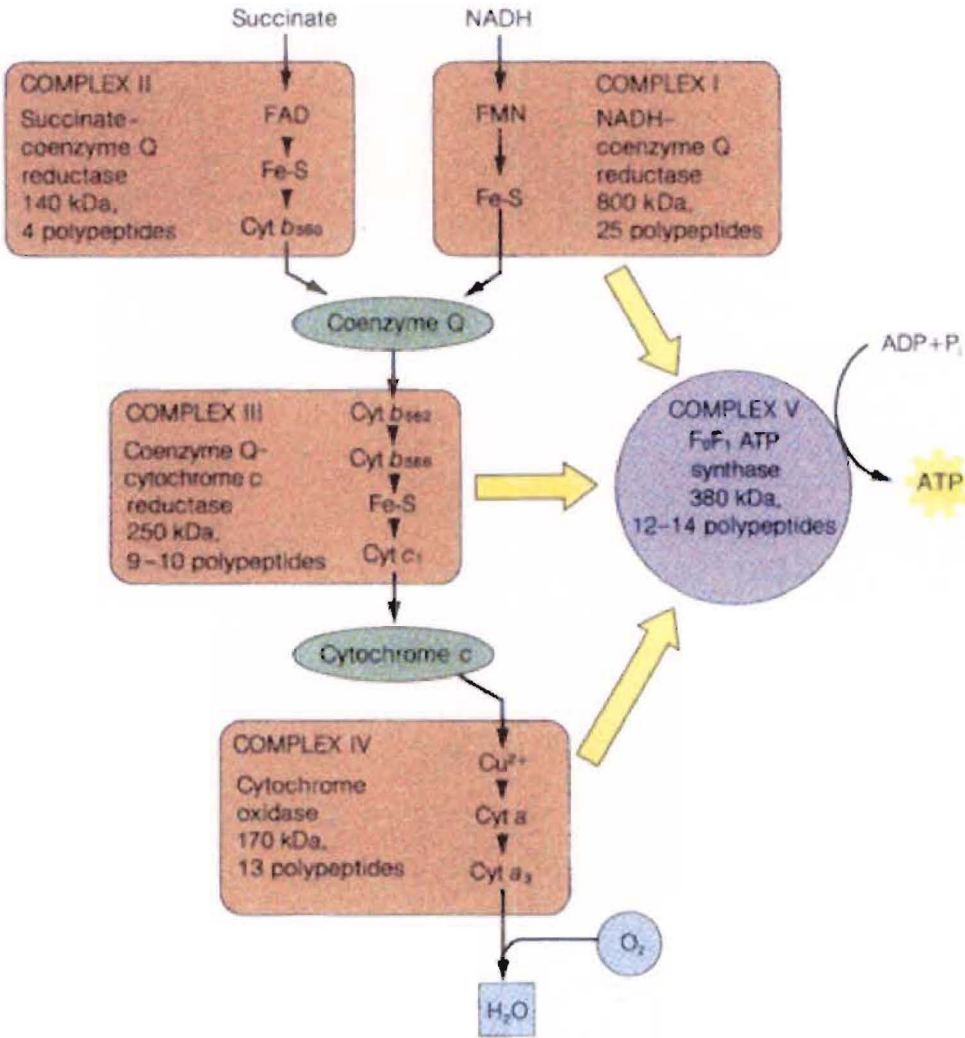
Many agents have been shown to interfere with the biosynthesis of CoQ, all of which act in the early stages of the pathway. Mevinolin, an inhibitor of HMG-CoA reductase, has also been shown to affect other enzymes in the mevalonate pathway and lowers the CoQ content of several tissues. Any inhibitors which decrease the farnesyl pyrophosphate pool also decrease the rate of CoQ synthesis.<sup>54</sup> No inhibitors of the final stages of CoQ synthesis are available, and the only inhibitors used clinically are HMG-CoA reductase inhibitors.<sup>54</sup>

A commonly encountered family of drugs affecting CoQ biosynthesis are the

statins. These drugs, e.g. lovastatin, are inhibitors of HMG-CoA reductase, and are commonly prescribed for hypercholesterolemia (high cholesterol). The transformation of HMG-CoA to mevalonate by HMG-CoA reductase is the rate limiting step in the synthesis of cholesterol making these inhibitors particularly effective. This step, however, is also crucial to CoQ synthesis, and thus patients receiving statin therapy often show significantly lowered plasma CoQ levels. The decrease in plasma CoQ levels with statin therapy can be prevented by concomitant administration of CoQ.<sup>53</sup> Despite this reduction in CoQ induced by statins, oxidation of LDL appears to be inhibited by statins, so no serious adverse effects have been observed in relation to CoQ deficiency.<sup>53</sup>

### 1.2.3 CoQ AND THE ELECTRON TRANSPORT CHAIN

Coenzyme Q is a coenzyme for at least 3 mitochondrial enzymes (complexes I, II, and III), as well as enzymes in other parts of the cell. The role of CoQ in the production of energy, in the form of ATP, via oxidative phosphorylation has been extensively studied (see figure 1.10).<sup>53</sup>

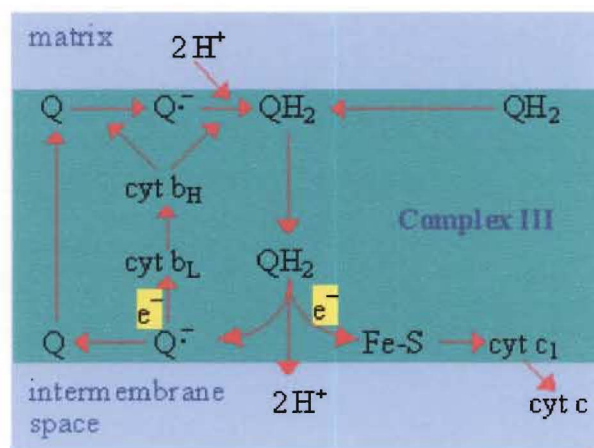


**Figure 1.10:** The electron transport chain of mitochondria

CoQ has a multifaceted function in energy transduction. It's role as a redox link between flavoproteins and cytochromes in the mitochondrial respiratory chain has been known since the mid 1960's.<sup>55</sup> CoQ acts as a traditional coenzyme to each dehydrogenase through a CoQ binding site on the enzyme.<sup>56</sup> Each binding site has individual characteristics and sequence of electron transfer, and one such system which has been well studied is that of succinate dehydrogenase.<sup>56</sup>

CoQ is involved in direct proton movement to establish a proton gradient across the membrane that can be coupled to ATP production.<sup>56</sup> First it gathers electrons from dehydrogenases for substrates oxidised by mitochondrial crista membranes.<sup>56</sup> The location of protonation, on the matrix or the inner side of the cristae membranes, lays the groundwork for proton transfer across the membrane.<sup>56</sup> The hydroquinone is reoxidised at specific sites on the quinol dehydrogenase, which transfers electrons from the quinone though a series of electron carriers.<sup>56</sup> Orientation of proton release

to the outside of the membrane is the final step in the chemiosmotic proton gradient formation.<sup>56</sup> This process is called the Q cycle (see figure 1.11).



**Figure 1.11:** The Q cycle\*

The Q cycle centres on the ability of the quinone moiety of CoQ to donate and accept two single electrons in association with its stepwise protonation and deprotonation.<sup>54</sup> One of the electrons involved is cycled through the semiquinone radical to the heme centre of cyt  $b_{566}$ , while the other electron is linearly transferred through the iron-sulfur centre of the Rieske protein and cytochrome  $c_1$ , to cytochrome oxidase.<sup>57,58</sup> The crucial intermediate in this process is the semiquinone radical ( $Q^{\bullet-}$ ), with the electron flux through this intermediate affecting the efficiency of energy gain through oxidative phosphorylation.<sup>54</sup> Between the primary quinone reduction site and subsequent quinol oxidation, the quinol diffuses through the lipid bilayer and back.<sup>56</sup> This implies that the efficiency of the process depends on the affinity and quality of quinone binding sites in the proteins, condition of the electron carrier elements and the ability of the quinone to move through the membrane.<sup>56</sup> The Q cycle is driven as long as electrons from the CoQ pool are transferable to the iron-sulfur protein.<sup>57</sup> If the oxidant side of  $CoQH_2$  is fully reduced, semiquinone pools with equilibrate at low levels.<sup>57</sup>

The concentration of CoQ in the membrane is not saturating for NADH dehydrogenase, that is the affinity of CoQ for the enzymes it interacts with is not high enough to saturate them at physiological concentration.<sup>59</sup> This implies that the velocity of the respiratory chain is strongly dependent on the CoQ concentration, and any event giving rise to an increase or decrease in the concentration of CoQ will

the flux of electrons through the respiratory chain.<sup>55,59</sup> Therefore, the administration of exogenous CoQ, which will increase the concentration of CoQ in the mitochondrial membrane, will increase the rate of the respiratory chain.<sup>56</sup> This process is limited by the low solubility of CoQ in the lipid membrane, making the greatest possible increase only 20-30% above the basal rate.<sup>56</sup> Any process which leads to a decrease in the mitochondrial total CoQ concentration, or excessively oxidising or reducing conditions in the mitochondrial CoQ pool, will lead to a skew in the CoQ:CoQH<sub>2</sub> ratio. The corresponding decrease in the concentration of either CoQ or CoQH<sub>2</sub> may inhibit mitochondrial proton movement and ADP phosphorylation, and produce serious physiological and medical problems.<sup>59</sup>

CoQ is also present in plasma membranes where its function relates to growth control and signal transmission.<sup>56</sup> The plasma membranes of all cells contain ligand-activated NADH oxidase that can transfer electrons across the membrane to activate cell signals and stimulate cell growth.<sup>56</sup> These signals are the same as those activated by other mitogenic ligands, such as an increase in internal pH or calcium ion concentration, activation of protein kinases and activation of the early protooncogenes.<sup>56</sup> As electron transfer across the plasma membrane induces proton release by the cell in the presence of CoQ, it is postulated that CoQ may be an agent for proton transfer in plasma membranes, as it is in mitochondria.<sup>60</sup> Most proton release activated by electron transfer at the plasma membrane is based on activation of the Na<sup>+</sup>/H<sup>+</sup> exchange, which can be inhibited by CoQ analogues,<sup>56</sup> but a small amount may be based on the redox cycling of CoQ.<sup>60</sup> CoQ may also control signalling across the plasma membrane as the redox change in lipophilic quinones may modify the function of the associated proteins leading to activation of protein kinases associated with the membrane.<sup>60</sup>

The involvement of CoQ in NADH-dependent redox chain also contributes to the acidification of the lumen by transfer of protons to the interior of the cristernae, in an analogous manner to proton movement in the mitochondria.<sup>57</sup>

### 1.2.4 CoQ AS AN ANTIOXIDANT

The second major role of CoQ is as an antioxidant. It has been shown that CoQ can reduce free radicals, which can cause damage to structural lipids or proteins in the membrane, not only in mitochondria but in any cellular CoQ containing membranes.<sup>56</sup> The reduced form of CoQ (CoQH<sub>2</sub>) acts as the reducing agent for this removal of free radicals or peroxides.<sup>56</sup> CoQH<sub>2</sub> can exert this free radical-scavenging activity and inhibit subsequent lipid peroxidation in liposomes, membranes, cells and lipoproteins.<sup>57</sup> This process is supported by the dehydrogenases in the membrane which can reduce CoQ to CoQH<sub>2</sub> thereby renewing the cellular supply.<sup>56</sup> Reduced CoQ is the only known lipid-soluble antioxidant which can be synthesised *de novo* in animal cells and for which there exists a mechanism of regeneration from the oxidised form.<sup>57</sup>

In addition to quenching free radicals itself, reduced CoQ can reduce the  $\alpha$ -tocopheryl radical to  $\alpha$ -tocopherol (the most active form of vitamin E), thereby recycling this important cellular antioxidant.<sup>57,59</sup> Reduced CoQ has been shown to preferentially reduce the  $\alpha$ -tocopheryl radicals over peroxy radicals, supporting this important function of CoQ in the regeneration of  $\alpha$ -tocopherol.<sup>57</sup> Purified human NADPH-cytochrome P<sub>450</sub> reductase can reduce the  $\alpha$ -tocopheryl radical in the presence of CoQ in an NADPH-dependent process.<sup>57</sup> Also CoQH<sub>2</sub> and the  $\alpha$ -tocopheryl radical are likely to occupy the same domain in membranes and collisions between the two would be facilitated by changes in the amphipathic character of the two lipids.<sup>57</sup>

Similarly, it has been shown that transplasma membrane electron transport, which depends on CoQ acts to reduce extracellular ascorbate free radical back to ascorbate, another important biological antioxidant.<sup>60</sup> Following exposure to a radical source, LDL deploys antioxidants which are consumed while opposing oxidative attack.<sup>59</sup> In this situation ascorbic acid is the first to intervene followed by CoQH<sub>2</sub>.<sup>59</sup> When LDL-ascorbic acid is exposed to radical attack peroxidation is under control as long as some CoQH<sub>2</sub> is present.<sup>59</sup> It is only when the CoQH<sub>2</sub> is almost entirely oxidised that lipid peroxidation occurs, when 95% of the initial vitamin E is still present.<sup>59</sup>

The bioenergetic and antioxidant roles of CoQ are not independent. Any condition of increased oxidative stress leads to enhanced involvement of CoQ as an



antioxidant which may decrease the critical availability of CoQ for oxidative phosphorylation.<sup>59</sup> In many disease states arising from a CoQ deficiency, both these roles are important.<sup>59</sup>

### 1.2.5 PROOXIDANT ACTIVITY

Under certain conditions the semiquinone radical which is an important intermediate in the Q-cycle (see section 1.2.3) may undergo autooxidation by reaction with molecular oxygen to produce the highly reactive superoxide radical.<sup>57</sup> This process, which is not expected during normal respiration, effectively converts CoQ from an antioxidant to a prooxidant supporting lipid peroxidation.<sup>57,58</sup> The reaction of superoxide radicals with CoQH<sub>2</sub> is also possible giving the semiquinone radical and hydrogen peroxide.<sup>57</sup>

The process of superoxide formation by the semiquinone radical requires the availability of protons, and there are two situations when this autooxidation is possible; if protons can penetrate into a disturbed membrane allowing reaction, or if the physical state of the membrane remains unchanged while mobility of the redox-cycling semiquinone is changed so it can come in contact with the aqueous phase of the polar headgroup section of the membrane.<sup>57,58</sup> Both of these situations may occur in response to various toxicological or pathophysiological events (e.g. ischemia), or during ageing.<sup>57,58</sup> The mitochondria from heart tissue of aged rats are active superoxide generators, and also exhibit decreased membrane fluidity and lower respiration control values suggesting increased membrane permeability for protons.<sup>57</sup> This prooxidant role of CoQ is also utilised by the cell, in the formation of superoxide radicals in neutrophils and lysosomal granules.<sup>57</sup>

### 1.2.6 CoQ AND AGEING

The antioxidant effect of CoQ is considered important in retarding ageing in view of the reported accumulation of free radicals attacking tissues of the elderly.<sup>55</sup> According to the free radical theory of ageing mitochondria are both the main source and target of oxygen free radicals.<sup>55</sup> A decrease in the activity of mitochondrial enzymes is suggested to result from somatic DNA mutations induced by oxygen free radicals, implying that the level of antioxidants in the cell may be critical to

preventing or retarding this free radical damage.<sup>55</sup> The incorporation of exogenous CoQ in the cell is postulated to limit the propagation of damaging free radicals from the cytosol to the mitochondria protecting against free radical damage.<sup>55</sup>

There is also a clear relationship between age and mitochondrial respiratory complex deterioration.<sup>59</sup> The age related decline of CoQ is greater than the age related decline of many of the other lipids.<sup>59</sup> Exercise may minimise the effect of this CoQ reduction, and in animal models a regimen of endurance training increased not only the concentration of CoQ in heart submitochondrial particles, but also the plasma levels of enzymes such as superoxide dismutase which are radical scavengers.<sup>59</sup>

### 1.2.7 CoQ AND DISEASE

A deficiency of CoQ has been observed in many diseased states, and supplementation with exogenous CoQ is suggested to be advantageous for a range of disorders from cancer and diabetes to heart disease and neurodegenerative disease. The part CoQ may play in these diseases hinges on its antioxidant, and electron carrier roles as described above.

#### Cardiovascular Disease

As discussed earlier, in 1972 Littaru and Folkers first documented a CoQ deficiency in patients with heart disease.<sup>52</sup> This deficiency could be due to impaired biosynthesis of CoQ, or accelerated catabolism, as an increased antioxidant commitment of CoQ may lead to accelerated consumption.<sup>59</sup> The basis for the therapeutic effects of CoQ may be due to the high levels of aerobic energy production in cardiac tissue,<sup>56</sup> or action as an antioxidant against the high levels of free radicals produced in many forms of cardiovascular disease.<sup>59</sup> In heart disease a deficiency of vitamin E leads to peroxidation of many lipids, including CoQ. Supplementation with H<sub>6</sub>CoQ<sub>4</sub> gave partial replacement of the endogenous pool of CoQ with restoration of respiratory chain activity.<sup>59</sup> Disturbance of energy production with consequent energy starvation of muscle cells has been proposed as a final common pathway in the progression of myocardial disease of various etiology.<sup>59</sup> An increase in the LDL:CoQ ratio in heart disease patients has been found to be more consistent than the corresponding increase in the total cholesterol:HDL cholesterol ratio which is a commonly used coronary risk factor.<sup>59</sup>

It was observed as early as 1977 that patients with hypertension exhibited low levels of CoQ, and supplementation showed a decrease in blood pressure associated with a reduction in peripheral resistance.<sup>61</sup> CoQ supplementation has also shown to be beneficial to patients suffering from angina<sup>53,62</sup> or arrhythmias.

Clinical trials have shown that in patients with congestive heart failure the administration of CoQ in conjunction with conventional therapy can reduce the risk of hospitalisation by 38%, and reduce the incidence of pulmonary edema by 60%,<sup>63</sup> as well as improving clinical symptoms in most patients.<sup>64</sup>

Cardiomyopathy is a state in which the muscle tissue of the heart has become damaged, enlarged or stretched leaving the muscle fibres weakened. This may be secondary to nutritional deficiencies, or brought on by longstanding excess alcohol consumption or infection, and tends to be associated with a major CoQ deficiency.<sup>53</sup> Whether CoQ deficiency is the cause or an effect of cardiomyopathy is still unclear.<sup>53</sup> Again with CoQ supplementation a vast improvement in symptoms, and myocardial mitochondrial function was observed in several clinical trials.<sup>65,66</sup>

When pump failure is due to ischemic heart disease, increased radical production might exist according to a well studied mechanism linking oxidative damage to ischemia-reperfusion.<sup>59</sup> Therapeutic uses of CoQ may also be related to the beneficial effects of antioxidants in this clinical syndrome.<sup>59</sup> In a rabbit model of ischemia-reperfusion, pre-treatment with CoQ gave a relative maintenance of tissue ATP and ATP generating capacity of the mitochondria together with smaller calcium overload.<sup>53,59</sup> These beneficial effects are similar to those seen with drugs such as propranolol (a beta-adrenergic receptor blocking agent) and verapamil (a calcium channel blocking drug).<sup>53</sup>

There are three sources of free radicals during myocardial ischemia; oxygen, neutrophils, and mitochondria.<sup>53</sup> Since skeletal muscle and myocardium are rich in myoglobin, the formation of hydrogen peroxide from ischemia-reperfusion may trigger myoglobin oxidation leading to ferryl myoglobin and further damage.<sup>59</sup> It has been shown that reduced CoQ slows the formation of ferryl myoglobin.<sup>59</sup> Reduced CoQ is instrumental in allowing heme to act as a device for removing hydrogen peroxide and other peroxides.<sup>59</sup> Mitochondria from untreated hearts can be transformed into superoxide generating machines via the autooxidation of semiquinone radicals upon exposure to the metabolic conditions of ischemia-reperfusion.<sup>57</sup> CoQ may have the ability to maintain the integrity of myocardial

calcium channels and potassium channels during ischemic insults.<sup>53</sup> It may therefore activate potassium channels similar to nicorandil, and modulate calcium channels resulting in decreased cellular calcium ion concentration and improved cardiac integrity during ischemia.<sup>53</sup> CoQ protects ischemic tissue from reperfusion damage by antioxidant membrane stabilising properties and free radical scavenging activity.<sup>53</sup>

### **Periodontal Disease**

Gingival tissue in patients with periodontal disease was shown to have decreased CoQ levels.<sup>59</sup> Oral administration of CoQ lead to increased levels of CoQ in affected tissues and a decrease in inflammation, while other periodontal assessments also showed improvement.<sup>59</sup>

### **Neurodegenerative Disease**

It is possible that mitochondrial defects play a key role in the pathogenesis of neurodegenerative diseases and it has been demonstrated that CoQ administration can increase brain mitochondrial concentrations in older animals.<sup>67</sup> CoQ is postulated to play a neuroprotective role, involving both improved mitochondrial function and antioxidant activity, and has efficacy in a number of animal models for neurodegenerative diseases.<sup>67</sup> In animal studies CoQ can protect against malonate and 3-nitropropionic acid neurotoxicity, which are models for Huntington's disease, and against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity which is consistent with potential therapeutic effects in Parkinson's patients.<sup>67</sup> CoQ has been shown to exert a biochemical improvement in Huntington's patients as assessed using MRI and is now in clinical trials.<sup>67</sup>

Patients with progressive muscular dystrophy also exhibited low CoQ levels and clinical trials involving such patients showed improvements in cardiac output in addition to increased exercise tolerance, and decreased fatigue.<sup>68</sup>

### **Diabetes**

Diabetes is a multifactorial disease associated with a number of different metabolic abnormalities. As CoQ plays a major role in the electron transport chain, which is important in carbohydrate metabolism, a deficiency of CoQ may have an adverse effect of glucose tolerance. Mitochondrial dysfunction and oxidative stress

have also been shown to participate in the development of diabetic complications, but the mechanism of their origin is unclear.<sup>69</sup>

A study involving rats with experimentally induced diabetes showed increased levels of lipoperoxidation and decreased mitochondrial levels of CoQ in the heart and liver.<sup>69</sup> A clinical study involving 39 diabetic patients also showed a consistent fall in fasting blood glucose levels and ketone body concentration in those patients receiving CoQ when compared with controls.<sup>70</sup>

## **Cancer**

CoQ has been postulated to reduce the cardiotoxicity of certain chemotherapeutic drugs. Studies have shown that the cardiac toxicity of doxorubicin (of the anthracyclin family of chemotherapeutics), postulated to be due to interference with mitochondrial electron transport in heart tissue, is decreased with CoQ supplementation in humans.<sup>59,71</sup>

In addition CoQ deficiencies have been noted in patients with varying forms of cancer including breast, lung, prostate, pancreatic, and colon cancer.<sup>72-74</sup> Supplementation of a group of breast cancer patients with CoQ plus vitamin C, vitamin E, beta carotene, selenium and omega-3 and omega-6 fatty acids in addition to chemotherapy showed no increase in metastases, and in 20% of cases partial tumour regression.<sup>75</sup>

## **Immune Disorders**

The administration of CoQ or a combination of CoQ and vitamin B6 has been shown to boost the immune system and may be useful in the treatment of AIDS and other infectious diseases.<sup>76</sup> Patients with HIV-infection have been shown to have a deficiency of CoQ, which became more severe with progression of the disease. Exploratory therapy with CoQ showed encouraging, and in some cases striking results.<sup>77</sup>

## **1.3 RESEARCH DESCRIBED IN THIS THESIS**

Due to the varied, and biochemically important, roles of vitamin K a fast and efficient method for assaying vitamin K levels in plasma could be applied widely in medicine. Several methods for measuring vitamin K by high-performance liquid

chromatography (HPLC) have been developed and these will be reviewed in chapter 2. The methods for the determination of vitamin K in various media published to date are labour intensive and used for research purposes only. For this reason the main goal of this study was to investigate methods for the precise and efficient assay of vitamin K in plasma. Two approaches were taken to this problem, the development of a new HPLC based assay for vitamin K (see chapter 2), and early investigations into the feasibility of an immunoassay for vitamin K.

The development of an immunoassay involves the production of monoclonal antibodies to a given antigen, in this case vitamin K (see chapter 4). Given that vitamin K is lipid soluble, and too small to evoke an immune response in a host organism, the first step in this process was attach an analogue of vitamin K to a suitable carrier protein (see chapter 3). This in turn required the design and synthesis of a series of side-chain functionalised analogues of vitamin K.

Due to the structural similarities between vitamin K and coenzyme Q, and the importance of CoQ in many biological processes, the potential use of an immunoassay for CoQ was also identified. Therefore, a series of analogues of coenzyme Q were also designed and synthesised (see chapter 5).

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## **CHAPTER TWO**

### **DEVELOPMENT OF HPLC ASSAY**

## 2.1 INTRODUCTION

The diverse biochemical roles of vitamin K, and its implication in disease states, has led to a large amount of research being devoted to the development of a simple and efficient assay for vitamin K in various media including plasma, tissue, foods and milk. There are several intrinsic difficulties in the development of such an assay. Vitamin K is sensitive to UV light and alkaline conditions, and is present in tissues and plasma at levels several-fold lower than the other fat-soluble vitamins.<sup>1</sup> Also measurement and isolation of the K vitamins is complicated by coeluting lipophilic material and the multiple forms of K that possess biological activity.<sup>1</sup>

Since the mid 1960's many different assay techniques have been applied either to the detection of vitamin K directly, or various markers for vitamin K deficiency in biological samples. Assays involving biological markers of vitamin K deficiency include; the detection of urinary  $\gamma$ -carboxyglutamic acid by HPLC, functional blood coagulation studies (including prothrombin time), the detection of protein induced by vitamin K absence or antagonism (PIVKA-II) – a measure of undercarboxylated prothrombin often by immunoassay, and more recently the detection of undercarboxylated osteocalcin as an indicator of vitamin K status in bone.<sup>1</sup>

The earliest method used for the determination of levels of vitamin K activity was the chick bioassay developed by Matschiner and Doisy in 1966.<sup>2</sup> This was essentially a qualitative index which, although used for all early investigations into vitamin K content of various foods, lacked precision.<sup>1</sup> As analytical techniques have improved over the last 35 years so have the methods used for vitamin K determination. Thin-layer chromatography<sup>3</sup> and gas chromatography<sup>4</sup> have both been utilised for vitamin K<sub>1</sub> analysis but these methods required large volumes of biological samples and multiple chromatographic steps. More recently, a mass spectrometry method combining mass-selective detection with the benefits of stable isotopes dilution for internal standardisation was developed.<sup>5</sup> This method for the quantitative determination of vitamin K<sub>1</sub> involves the extraction of vitamin K from human plasma and derivatisation to the heptafluorobutyryl ester after reduction of one carbonyl group with zinc metal.<sup>5</sup> This

method allows measurement in small quantities of plasma and has a detection limit of 2.0 pg/ml of plasma (4.4 pM).<sup>5</sup>

With the introduction of high performance liquid chromatography (HPLC) a variety of different analytical procedures for the determination of vitamin K in various matrices have been developed. The first HPLC methods developed in the early 1980's used UV detection for the identification and quantitation of vitamin K<sub>1</sub>.<sup>6-9</sup> The first determination of vitamin K in human milk was achieved by Haroon *et al* in 1982 using a multistep chromatographic process.<sup>9</sup> Extraction of the sample was followed by adsorption chromatography and semi-preparative HPLC to remove the contaminating lipids, before analytical HPLC with UV detection.<sup>9</sup> Quantitation was achieved by the use of an internal standard, in this case vitamin K<sub>1</sub>-epoxide.<sup>9</sup> This method was successfully used to quantify vitamin K<sub>1</sub> in human milk, cows milk and selected infant formulas, but was unable to identify the presence of menaquinones in either human or cows milk.<sup>9</sup>

In the same year the first report of the use of HPLC for the determination of vitamin K levels in human blood was published.<sup>8</sup> Again this was a multidimensional analysis involving normal phase chromatography for removal of polar lipids followed by reverse phase (RP) analytical HPLC with UV detection.<sup>8</sup> This method was able to detect vitamin K to levels of 0.5 ng/ml of serum (1.1 nM) with a detection limit of 400-550 pg per injection.<sup>8</sup>

Another commonly used detection technique for vitamin K assays was electrochemical detection, with one of the earliest examples of this being reported by Ikenoya *et al* in 1979.<sup>10</sup> Many early assays using this technique experienced problems with increased background currents due to the reduction of interfering compounds such as oxygen.<sup>1</sup> This problem was overcome by Haroon *et al*<sup>11</sup> by employing a dual electrode system in redox mode, with the quinone being reduced at the first electrode and reoxidized at the second 'detector' electrode. This system demonstrated improved sensitivity over earlier methods with a detection limit of 100 pg per injection.<sup>11</sup> Further improvements on this technique, for the determination of sub-normal plasma levels of vitamin K in plasma, were later established by Hart *et al*.<sup>12</sup> This method halved the detection limit of previous assays to 50 pg per injection.

Vitamin K<sub>1</sub> itself does not fluoresce but its reduced hydroquinone form is highly fluorescent. By exploiting this fact Langenberg and Tjaden<sup>13</sup> developed an HPLC system

using dual electrode reduction for post-column derivatization of vitamin K<sub>1</sub>, followed by fluorescence detection. The sensitivity of this general method was limited by the fact that only 40-60% of the quinone injected was reduced to the fluorescent hydroquinone.<sup>14</sup>

During investigations into the use of a zinc packed column as an oxygen scrubber for use in the electrofluorimetric system it was observed that zinc metal, in the presence of zinc ions, was able to reduce vitamin K to its fluorescent hydroquinone form.<sup>1</sup> With a mobile phase of 10mM zinc chloride, sodium acetate, and acetic acid in 2:8 dichloromethane:methanol, 95% reduction of the injected vitamin K was observed upon reaction with metallic zinc.<sup>15</sup> This method had several advantages over previous systems as it required only a single chromatographic step, and utilised smaller volumes of plasma (0.5-1 ml).<sup>15</sup> Although the lower detection limit for this assay was 50 pg/ml of plasma (0.1 nM), the extraction procedure described was complicated, and did not lend itself to use in high-throughput routine analysis. This general HPLC system has since been adapted for use by many laboratories and is still used today for the analysis of vitamin K<sub>1</sub> levels in plasma,<sup>15-17</sup> tissue,<sup>16</sup> and food samples,<sup>16,18-21</sup> as well as the determination of menaquinones<sup>17,19</sup> and vitamin K<sub>3</sub> (menadione) in various matrices.<sup>22</sup>

As well as zinc metal reduction systems, many other methods for the reduction of vitamin K to the hydroquinone have been used in conjunction with fluorescence detection in assay systems for the K vitamins. Lambert *et al* developed a method utilising temperature-dependent wet-chemical post-column reduction.<sup>23</sup> In this method tetramethylammonium octahydridotriborate, present in the mobile phase, reduced vitamin K only upon heating in a post-column reaction coil to 80 °C.<sup>23,24</sup> This process was used to measure vitamin K<sub>1</sub> levels in serum, and was shown to have a minimum detectable level of 50 pg/ml of serum (0.1 nM).<sup>23,25</sup>

Photochemical reduction has also been employed for the post-column derivatization of vitamin K before fluorescence detection.<sup>26</sup> This is a reagent free process in which the quinones are photoreduced via hydrogen abstraction from the HPLC mobile phase in a post-column photoreactor.<sup>26</sup> Using this method vitamin K<sub>1</sub> was detected in plant extracts without prefractionation.<sup>26</sup>

As well as zinc a number of other metal catalysts have been investigated for the reduction of vitamin K 'on-line'. M Shino in 1988 published an HPLC assay utilising platinum oxide in the presence of hydrogen for the reduction of vitamin K.<sup>27</sup> After an

investigation of several different possible catalysts MacCrehan *et al* used 10% platinum on alumina, with an alcohol mobile phase, for a similar purpose.<sup>28</sup> Finally Usui *et al* have determined vitamin K<sub>1</sub> and menaquinone levels in human liver to subnanogram levels using a platinum black catalyst and alcohol mobile phase, coupled with fluorescence detection.<sup>29,30</sup> In the methods of MacCrehan and Usui it is the alcohol which, upon oxidation by the transition metal catalyst, acts as the reducing agent for the reaction.

## 2.2 OXIDATION OF METHANOL BY METAL CATALYSTS

Based upon the methods of Usui and MacCrehan,<sup>28-30</sup> the oxidation of methanol by various metal catalysts, under conditions suitable for an HPLC assay, was investigated.

The oxidation of methanol by a metal catalyst has been extensively studied due to its application in combustion engines. Several different metal catalysts have been investigated, including platinum, palladium, silver, and metal oxides.<sup>31-37</sup> The kinetics and mechanism of this reaction have been studied, usually in the gas phase at raised temperatures and pressures.<sup>32,33,35,38,39</sup> Oxidation of methanol vapour over platinum has been shown to occur via two parallel mechanisms, one inhibited by oxygen and one which is not. The uninhibited reaction is thought to involve the breaking of a metal-oxygen bond as the rate-determining step.<sup>32</sup> The dehydrogenation of surface adsorbed methanol on pure platinum was shown to be energetically favourable by Ishikawa *et al*, with the first step in this process being the removal of a hydrogen atom from the methyl group.<sup>40</sup>

In the case of silver all studies have been carried out at high reaction temperatures (900 K), and it was found that under these conditions the silver surface is restructured and a tightly held oxygen species is formed on the surface. Boa *et al* showed that it is this oxygen species which is subsequently involved in the oxidation of methanol to formaldehyde.<sup>35</sup> The use of copper oxide catalysts is thought to proceed via a mechanism in which the oxidation of adsorbed surface structures, formed upon interaction between methanol and the catalyst surface, is the rate-determining step.<sup>39</sup>

The combination of methanol and a metal catalyst has also been used as a reduction system for the many different compounds including quinones, and nitro derivatives of

polynuclear aromatic hydrocarbons (nitro-PAH). Tejada *et al*<sup>41</sup> used a column of alumina coated with platinum and rhodium, in combination with a methanol/water mobile phase to reduce nitro-PAH to primary aromatic amines. This process was shown to be catalytic with no consumption of the platinum/rhodium surface, and required no modifiers in the mobile phase. Usui *et al* have used a similar system for the reduction of vitamin K.<sup>30</sup> In this system the methanol is oxidised by the metal catalyst, at the same time acting as a reducing agent for vitamin K.

### 2.2.1 EFFECTIVENESS OF METAL CATALYSTS

The first step of our research involved an investigation of the relative effectiveness of various methanol/metal catalyst systems towards the reduction of quinones using vitamin K. In total eight different metal catalysts were investigated, including five different forms of platinum, silver powder, copper (I) oxide, and palladium black. Each catalyst was packed into a 20 mm x 2 mm refillable guard column and flushed with methanol at a flow rate of 0.5 ml/min for at least 1 hour. It must be noted that caution is required in the packing of the platinum reactors as methanol is readily ignited by air in the presence of platinum. A sample of 0.5  $\mu$ M standard solution of vitamin K<sub>1</sub> was injected into a flow of 0.1 ml/min methanol. This was passed directly through the metal catalyst packed guard column to the fluorescence detector with excitation wavelength of 249 nm and emission wavelength of 408 nm. The 'peak' obtained plateaued at maximum fluorescence due to the slow flow rate, and this height was used to determine the level of reduction of vitamin K<sub>1</sub> by each catalyst system. The results of this experiment are given in table 2.1. When using fluorescence detection, the exact fluorescent response is an arbitrary response individual to a given detector, so it is invalid to compare exact values with any literature results. These values may, however, be used to calculate relative results for the different metal catalysts, which will be constant between different detectors.



Reduction Catalyst	Fluorescence	% Fluorescence
Platinum black	580	100
Platinum black (new)	475	82
Platinum powder (0.27-0.47 micron)	495	85
Platinum powder (0.5-1.2 micron)	355	61
Platinum on carbon	0	0
Platinum on alumina	290	50
Silver powder	10	2
Copper (I) oxide	5	1
Palladium black	15	3
None	0	0

**Table 2.1:** Reduction efficiency of various catalysts, absolute fluorescence observed and relative efficiency upon comparison with reference column (platinum black)

From these results it can be seen that the platinum compounds had superior reducing efficiency when compared to the other metal catalysts tested. The only exception to this was platinum on carbon, which showed no reduction activity. In this case it is postulated that the vitamin K is adsorbed onto the porous carbon surface. Analysis of 10% platinum on carbon by MacCrehan *et al.* gave the same result in a similar study.<sup>28</sup> In their study, which compared the reducing efficiencies of 5% platinum on alumina, 5% rhodium on alumina, 5% palladium on carbon, 10% platinum on carbon, platinum (IV) oxide, and 10% platinum on alumina, MacCrehan found that 10% platinum on alumina was the most efficient reduction catalyst.<sup>28</sup> We have shown above that both platinum black and platinum powder can out-perform 10% platinum on alumina in the reduction of vitamin K with methanol.

Palladium is another transition metal that has been used in the catalysis of methanol oxidation, and hence was expected to effect the reduction of vitamin K<sub>1</sub>. Upon testing, however, palladium black gave a much lower fluorescent response than the platinum compounds and was not used in further investigations. It was shown by Ionescu *et al* that the oxidation of methanol on palladium catalysts displays oscillating behaviour possibly due to the limited range of methanol concentrations on the catalyst surface leading to a

critical concentration of active species being obtained.<sup>34</sup> This effect may explain the low reduction of vitamin K by palladium black/methanol system, if critical concentrations of these active species were unable to be attained.

The failure of silver powder to effect any oxidation of methanol under the conditions studied can be explained, as silver catalysts have previously been used for methanol oxidation at high temperature, and have not been used for room temperature studies.<sup>35</sup> Similarly, copper oxide catalysts have previously been studied at temperatures of 150-270 °C and not at room temperature, explaining the low reduction of vitamin K observed.<sup>39</sup>

Although platinum black showed the most efficient reduction of vitamin K to the hydroquinone, results were inconsistent for some packings. In one instance the guard column had been cleaned with nitric acid, washed with water and methanol, and dried prior to packing with platinum black. This column gave consistently low fluorescence results and it is postulated that trace amounts of nitrates from the washing may interfere with the platinum black surface hindering methanol oxidation.

As well as giving a high fluorescent response the platinum black system does not lose reduction efficiency over time. The zinc metal reduction system used in many laboratories requires repacking of the zinc filled catalyst column after approximately four hours of use. However, upon exposure to an alcohol mobile phase, the lifetime of the platinum black catalyst appears indefinite.

In a study of platinum black from various different suppliers (Aldrich, Merck and Acros), we have shown that consistent results are observed after thorough washing with methanol. The absolute fluorescence results obtained varied by only 5%, so catalyst source does not appear to be important (see table 2.2).

Supplier	Fluorescence
Aldrich	500
Acros	470
Merck	470

**Table 2.2:** Comparison of platinum black sourced from different suppliers

The results of this study confirm the findings of MacCrehan *et al*<sup>28</sup> and expand on these. It has been shown that platinum catalysts are the most efficient for use in the

described reduction system, with platinum black and platinum powder both producing high levels of fluorescence with vitamin K. As platinum black showed the greatest reduction of vitamin K this was used in all further investigations. Platinum black sourced from three different commercial suppliers showed little variation (5%) in fluorescent response, implying that the source of this catalyst is not important.

### 2.2.2 EFFICIENCY OF METAL CATALYST COLUMN

As the efficiency of freshly packed platinum black catalyst columns was found to vary on occasion, the efficiency of each catalytic column was tested before use. The first platinum black column used, which produced the greatest fluorescent response with vitamin K, was used as a reference column for the testing of all other catalytic columns. Upon placing another catalytic column in series with this reference column, no increase in fluorescent response was observed. As this was a consistently reproducible observation it was postulated that the reference column was acting at 95-100% reduction efficiency. The reduction efficiency of any given catalytic column was tested by either of the following methods.

#### METHOD 1

A sample of a 0.5  $\mu\text{M}$  standard vitamin K<sub>1</sub> solution was injected into a mobile phase of 100% methanol with a flow rate of 0.5 ml/min. This was passed directly through the catalytic column being tested, and onto a RP HPLC column for separation of the resulting quinone and hydroquinone. The reference column was positioned between the HPLC column and fluorescence detector. The reference catalytic column was necessary to afford reduction of any quinone not reduced by the test column. The ratio of peak area of the hydroquinone (retention time 6.0 min) to the total peak area of hydroquinone and quinone (retention time 21.3 min), was calculated as the reduction efficiency of the catalytic column.

## METHOD 2

Alternatively, a sample of a 0.5  $\mu\text{M}$  standard vitamin  $\text{K}_1$  solution was injected directly into a RP HPLC column and through the catalytic column of interest, before detection via fluorescence. This process was then repeated after exchanging the test catalytic column for the reference column. The ratio of the peak areas for these two runs was then used to determine the reduction efficiency of the test column.

Both of these methods were used on any given catalytic column and the results compared. An example of the results obtained is outlined below in table 2.3. The reduction efficiencies for the test column in this case are 27% by method 1 and 34% by method 2.

Method	Column	Peak area
1	Test	62.4
	Test + Reference	230.0
2	Test	86.2
	Reference	250.0

**Table 2.3:** Results for reduction efficiency of test column by two methods

When a freshly packed catalytic column was found to have a reduction efficiency of less than 80% after thorough washing with methanol, attempts were made to increase the catalytic activity. This was done via washing with an ethanolic solution of sodium borohydride. A mobile phase of 100% methanol was pumped directly through the catalytic column at a flow rate of 0.1 ml/min. Successive injections of 400  $\mu\text{L}$  of the ethanolic sodium borohydride solution were then injected into the mobile phase at 3-minute intervals. This process was repeated until a total of two to three millilitres of sodium borohydride solution had passed through the catalytic column, and was followed by thorough washing with 100% methanol. Upon retesting the reduction efficiency of a treated column by method 2 (above), increased reduction efficiency was initially observed. However, over time, and with continued use, the efficiency of treated catalytic columns slowly decreased, until finally returning to the pre-treatment level. From this result it appears that a second reduction mechanism was taking place after treatment, which was responsible for the observed transient increase in reduction efficiency. It is postulated that an 'activated hydrogen species', originating from the borohydride wash, is

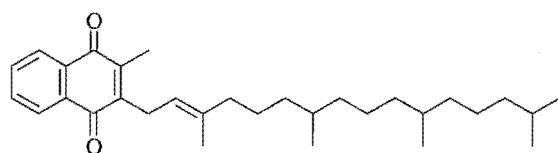
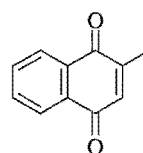
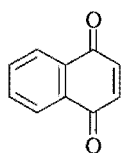
co-ordinating to the surface of the platinum black in the catalytic column. It is this 'activated hydrogen species' that is then responsible for the increased reduction of vitamin K to its hydroquinone form. This theory is supported by experiments carried out involving acid washing of the treated columns. When a borohydride treated column was washed with a 0.5% aqueous solution of trifluoroacetic acid following borohydride treatment, no significant increase in reduction efficiency was observed. This could be due to the removal of such an 'activated hydrogen species' from the catalytic column during the acid wash. A control experiment carried out by washing a catalytic column with only trifluoroacetic acid showed that this process itself, when followed by thorough washing with methanol, had no effect on the efficiency of reduction.

Two simple methods to test the efficiency of a specific catalytic column have been developed, and shown to give comparable results. Attempts to increase the efficiency of platinum reactors that were showing below 80% reduction on comparison with a reference column gave only a transient increase. However, conditions leading to possible loss of activity, e.g. exposure to nitrates, have been identified.

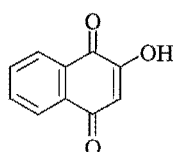
### 2.2.3 GENERALITY OF REDUCTION SYSTEM FOR VARIOUS QUINONES

This study aimed to establish the range of quinones that were effectively reduced by the methanol/platinum black system developed above. The extent of reduction was established by the level of increase in fluorescence of a standard quinone solution upon treatment with the reducing system, assuming the resulting hydroquinone form is more fluorescent than the quinone itself. Standard solutions of each of the quinones for this investigation (see figure 2.1), were prepared in ethanol at a concentration of 0.5  $\mu\text{M}$ . This was injected into a mobile phase of methanol at 0.1 ml/min. The quinone was then passed through a platinum black catalyst column and the fluorescent response recorded (c.f. method used for assessment of metal catalysts). During the time period of maximum fluorescent response a scan was run over a series of wavelengths in order to determine either the optimum excitation or emission wavelength for each quinone (see figure 2.2). Once these optimum values were established, each quinone was then retested at these optimal wavelengths to determine the level of reduction by the platinum black column. A control experiment run in the absence of any catalyst column was carried out for each

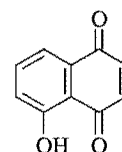
quinone to detect any native fluorescence. Any increase in peak height between the optimal wavelength run and the control experiment was interpreted as an indication of the level of reduction of each quinone.

Vitamin K<sub>1</sub>Vitamin K<sub>3</sub>

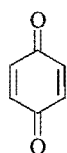
1,4-Naphthoquinone



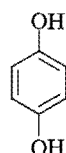
2-Hydroxy-1,4-Naphthoquinone



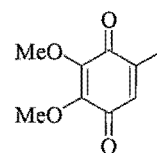
5-Hydroxy-1,4-Naphthoquinone



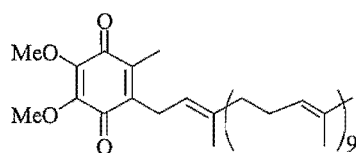
Benzoquinone



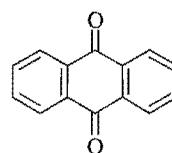
Hydroquinone



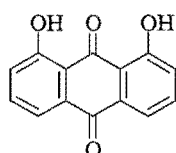
2,3-Dimethoxy-5-Methylbenzoquinone



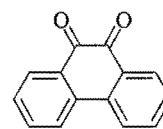
Coenzyme Q-10



Anthraquinone

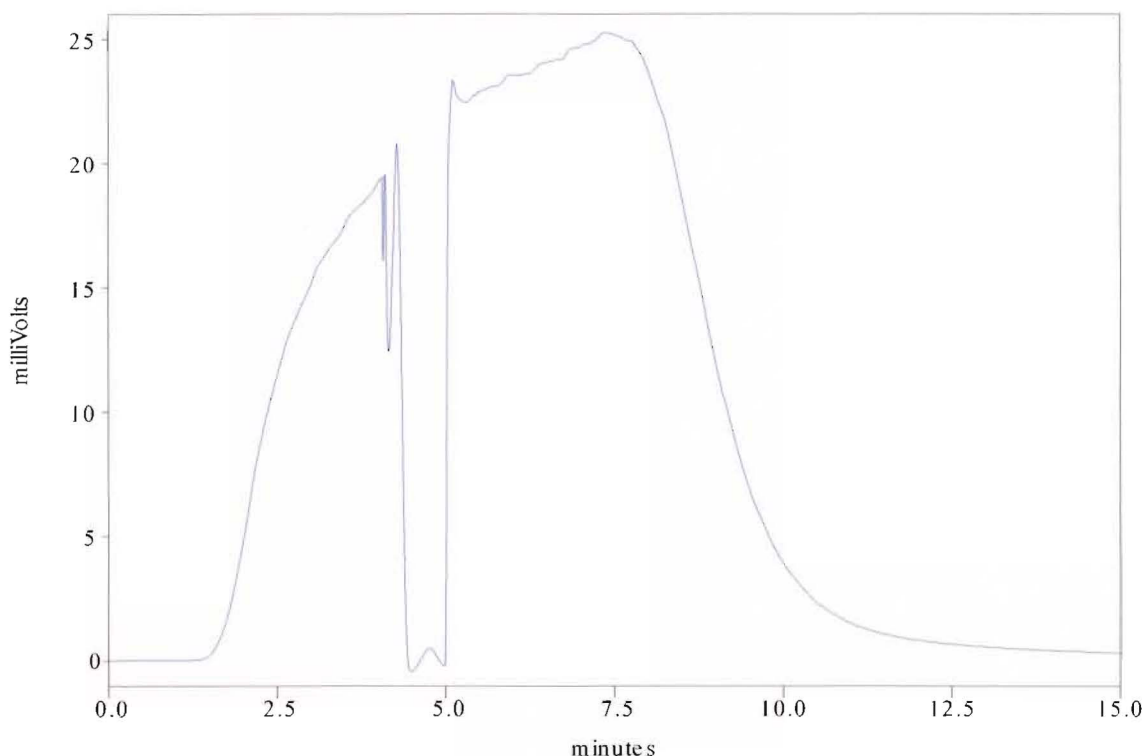


1,8-Dihydroxyanthraquinone



9,10-Phenanthraquinone

**Figure 2.1:** Range of quinones investigated in this study



**Figure 2.2:** HPLC trace showing the scan over excitation wavelengths 200-320 nm, performed during fluorescence of 2-hydroxy-1,4-naphthoquinone.

The results obtained from this study are outlined in table 2.4. It can be seen that not all of the quinones tested were reduced by the platinum black/methanol system. The K vitamins, 1,4-naphthoquinone and 2-hydroxy-1,4-naphthoquinone were all reduced to a more fluorescent form, as shown by the large increase in fluorescence observed in the presence of the platinum catalyst. Interestingly 5-hydroxy-1,4-naphthoquinone shows only a modest increase in fluorescence, which implies that the position of the hydroxyl group can have a significant effect on the reduction potential of these compounds. The benzoquinones, 2,3-dimethoxy-5-methylbenzoquinone (CoQ-0) and coenzyme Q-10, showed a slight increase in observed fluorescence in the presence of platinum black, although not as large as in the case of the K vitamins.

The three-ring anthraquinone system showed a similar level of increase in fluorescence in the presence of platinum black to the benzoquinones, but 1,8-dihydroxyanthraquinone exhibited no detectable increase in fluorescence. 9,10-

Phenanthraquinone was, however, efficiently reduced as shown by the large increase in fluorescence observed in the presence of platinum black. This increase was of the same order of magnitude as observed for the naphthoquinones.

Hydroquinone was run as a control experiment, as in this case the quinone is already in the reduced form before introduction into the system. The levels of fluorescence in the presence and absence of the platinum catalyst were the same, as expected. This control experiment is useful in ruling out the possibility that the reduction system produces any by-products, which may quench fluorescence.

Quinone	Peak height without Pt black (mV)	Peak height in presence of Pt black (mV)	$\lambda_{\text{ex}}$ (nm)	$\lambda_{\text{em}}$ (nm)
Vitamin K <sub>1</sub>	0	426	249	408
Vitamin K <sub>3</sub>	17	162	240	428
1,4-Naphthoquinone	4	164	242	418
2-Hydroxy-1,4-Naphthoquinone	9	250	242	436
5-Hydroxy-1,4-Naphthoquinone	9	27	309	359
Benzoquinone	7	11	278	325
Hydroquinone	37	39	278	325
2,3-Dimethoxy-5-Methyl- benzoquinone	49	63	284	310
Coenzyme Q-10	6	21	274	297
Anthraquinone	10	34	#	#
1,8-Dihydroxyanthraquinone	5	5	*	*
9,10-Phenanthraquinone	5	295	253	424

**Table 2.4:** Reduction of quinones to fluorescent hydroquinones with platinum black and methanol.

\* Wavelength data not obtained as no reduction occurred. # Wavelength data not obtained.

This investigation has shown that the methanol/platinum black reduction system is able to reduce a variety of different quinones, including selected benzo-, naphtho-, and anthraquinones. The ability of this system to reduce quinones of biochemical



importance, e.g. vitamin K and coenzyme Q, suggests that it may be used to establish an HPLC based assay system for these compounds.

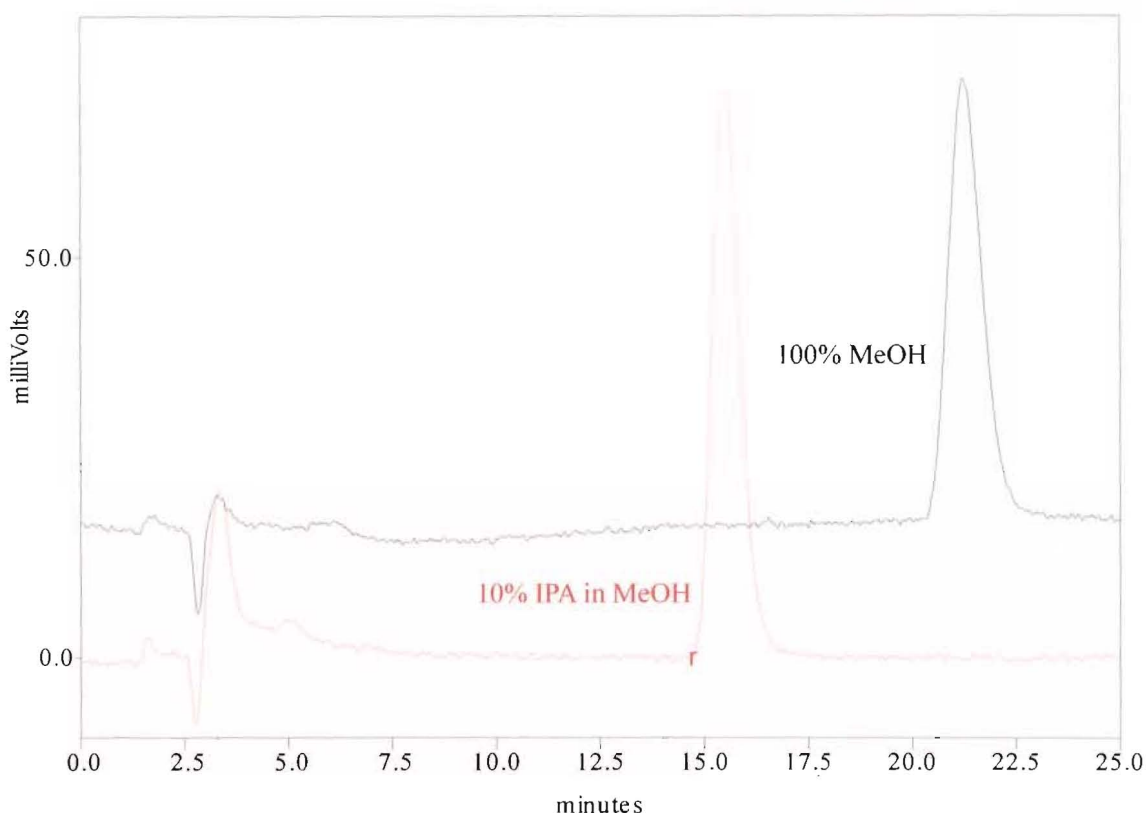
#### 2.2.4 EFFECT OF SOLVENT ON REDUCTION SYSTEM

Having established the effectiveness of the platinum black/methanol reduction system for an assay of various quinones, the effect of the addition of co-solvents and buffers to the mobile phase was investigated. Ideal assay conditions would exhibit a short retention time and high fluorescence. In practise, however, a compromise between these factors must be reached, while achieving separation of all components of a sample mixture.

Using a reverse-phase analytical HPLC column and platinum black catalytic column, the effect of a variety of different solvents on the reduction system was assessed. Any changes in the retention time and peak area of a vitamin K standard were recorded. A flow rate of 0.5 ml/min was used throughout this study, and the system was equilibrated for at least 30 minutes with each mobile phase before testing.

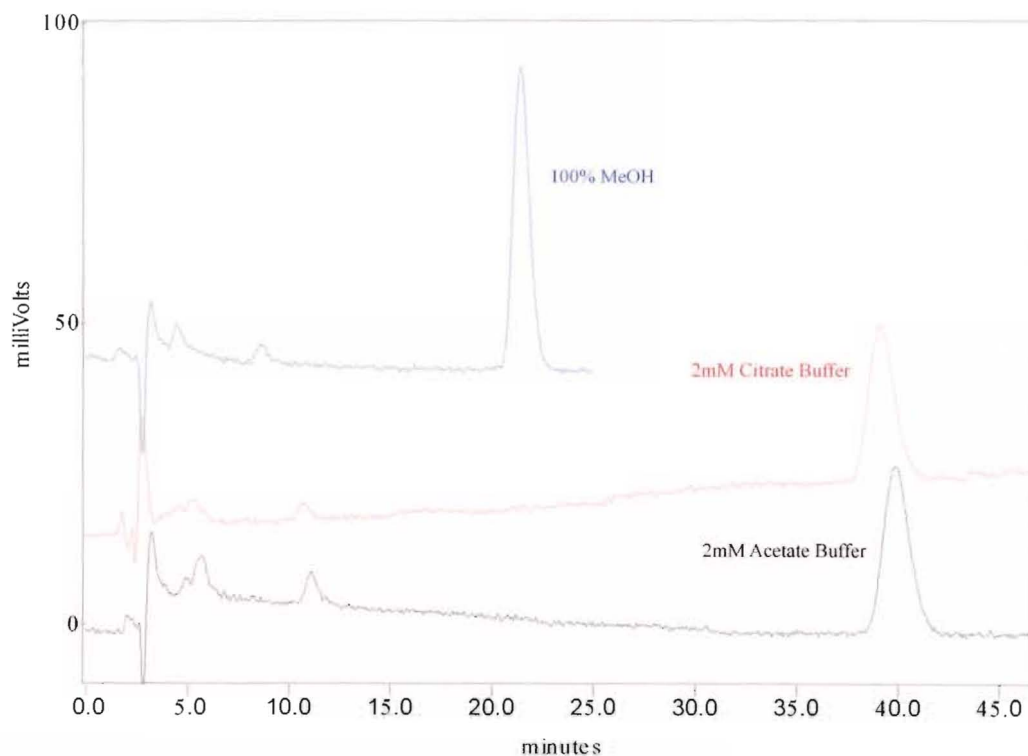
It was shown initially that the presence of an alcohol in the mobile phase was necessary for reduction to occur, as the absence of an alcohol gave no peak for a vitamin K standard solution. A series of different alcohol mobile phases were investigated. The order of reducing ability for these alcohols, i.e. how readily they were oxidised by platinum black, was methanol>ethanol>isopropyl alcohol>butanol>ethanediol. Taking this into account an investigation of various mixed alcohol mobile phases followed.

By increasing the proportion of ethanol in a methanol mobile phase the retention time of vitamin K<sub>1</sub> was found to decrease, but the observed peak area also decreased. The addition of up to 10% isopropyl alcohol to a methanol mobile phase had the effect of decreasing the retention time with no significant effect on the peak area observed (see figure 2.3). The addition of greater proportions of isopropyl alcohol showed a further decrease in retention time, but also led to an increase in operating pressure of the HPLC system. Adding up to 10% heptane to a methanol mobile phase gave an increase in the peak area with decreased retention time. However, this is at the limit of miscibility of these two solvents, which in some instances led to the development of two phases within the HPLC system.

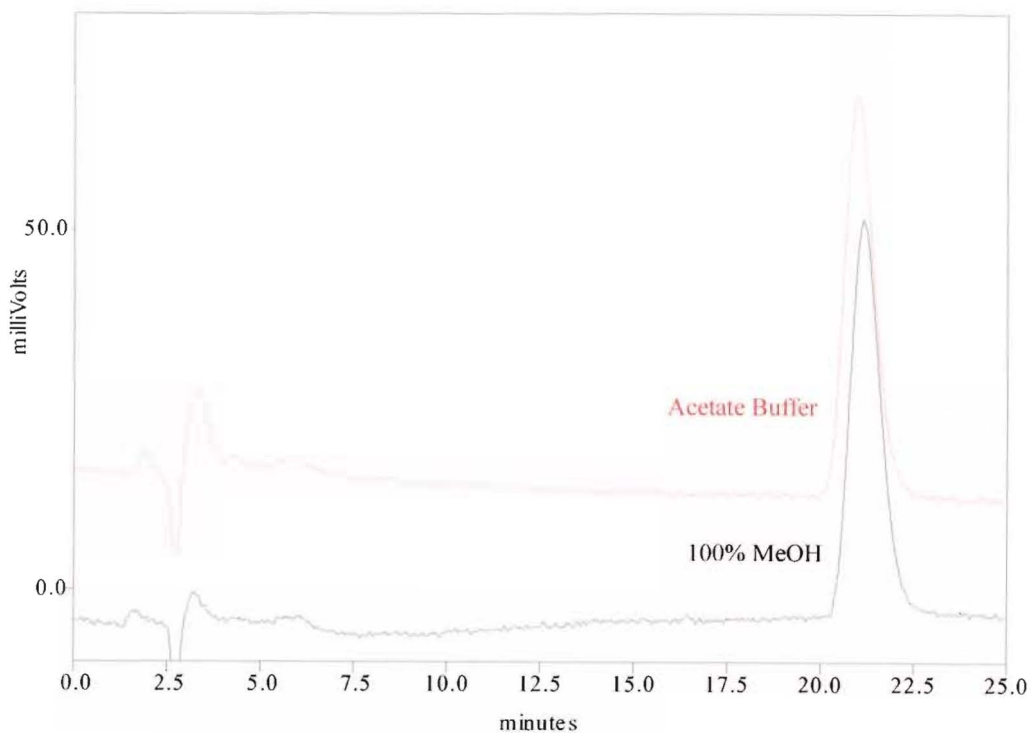


**Figure 2.3:** HPLC traces of standard vitamin K solutions with mobile phase of 100% methanol and 10% IPA in methanol

The inclusion of trace amounts of water (2%) in a methanol mobile phase gave a significant increase in retention time, with the vitamin K<sub>1</sub> peak moving from 21.0 minutes to 38 minutes. The same results were observed with a mobile phase of 2% aqueous acetate or citrate buffer (concentration 2mM) in methanol (see figure 2.4). This result implied that any change in the retention time and peak area of vitamin K<sub>1</sub> was due to the presence of water and not the buffer ions. This was verified by preparing a 2 mM acetate buffer in methanol, so no water was present in the mobile phase (see figure 2.5). The HPLC chromatogram obtained in the presence of the electrolytes was identical to that observed for a 100% methanol mobile phase showing no advantage in the inclusion of a buffer in the mobile phase.



**Figure 2.4:** HPLC traces of a standard vitamin K solution with three mobile phases; 100% methanol, 2mM aqueous citrate buffer in methanol, and 2mM aqueous acetate buffer in methanol



**Figure 2.5:** HPLC traces of standard vitamin K solution with 100% methanol a 2mM acetate buffer prepared in methanol.

Addition of some solvents to the mobile phase led to a decrease in the efficiency of the platinum black column. The study of an acetonitrile/methanol mobile phase showed that retention time increased with the amount of acetonitrile added and the peak area was at a maximum with a 50% acetonitrile/methanol solution. It was observed, however, that acetonitrile slowly poisoned the platinum black catalyst, although this could be reversed with thorough washing with 100% methanol. The investigation of a tetrahydrofuran/methanol mobile phase gave decreased retention time and peak area with an increasing proportion of THF. Again a slow poisoning of the platinum black catalyst was observed in the presence of THF, and this poisoning was reversible with extensive washing with 100% methanol. The poisoning effects of acetonitrile and THF have previously been documented by MacCrehan *et al* in their investigation of a catalytic reduction system using 10% platinum on alumina, however in this case presence of these solvents was also shown to completely prevent reduction.<sup>28</sup> When dichloromethane was added to the mobile phase the platinum black column was irreversibly poisoned. It is postulated that the dichloromethane altered the surface of the platinum black, no longer allowing methanol oxidation to occur.

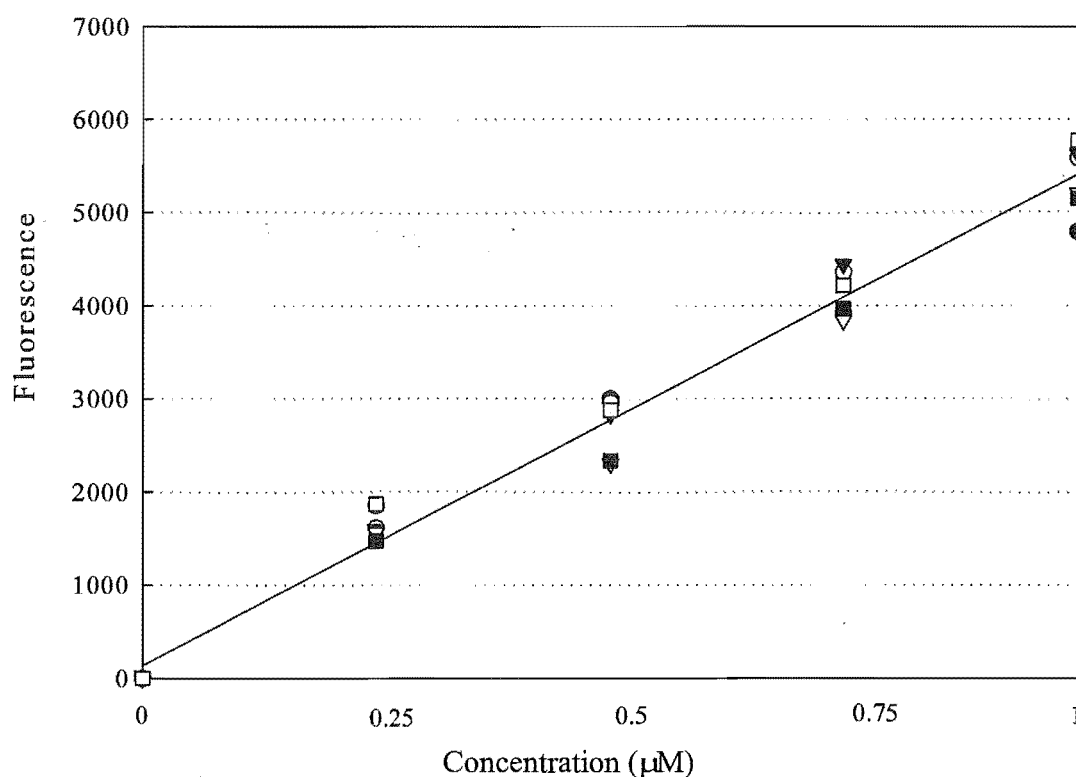
The most efficient solvent for reduction of quinones, as observed in this study, was 100% methanol, with the use of mixed alcohol mobile phases available to alter the retention time of the quinone on the RP HPLC column. The addition of water, or an aqueous buffer, to the mobile phase gave a vast increase in retention time, while adding acetonitrile, THF or dichloromethane was found to have a detrimental effect on the catalytic column. Given these results, 100% methanol was used as the mobile phase in all further investigations.

#### 4.2.5 LINEARITY OF FLUORESCENCE

In order to quantify vitamin K levels in any given sample, the behaviour of fluorescence over a given concentration range must be known. A series of standard solutions of vitamin K1 were prepared in ethanol to give the following concentrations; 0.25  $\mu\text{M}$ , 0.5  $\mu\text{M}$ , 0.75  $\mu\text{M}$ , and 1  $\mu\text{M}$ . A sample of each standard solution was injected into a mobile phase of 100% methanol at a flow rate of 0.5 ml/min. This was passed through a RP analytical column and reduced by the platinum black/methanol system

before detection via fluorescence. The peak area observed for each standard solution was recorded. Each of the four standard solutions were prepared and tested on four separate occasions to assess the reproducibility of the data obtained. Figure 2.6 summarises the results obtained, and shows that the relationship between concentration and peak area is linear over the concentration range used. Therefore, the addition of standard concentrations to each assay run would allow the establishment of a calibration curve for that run, and quantitation of vitamin K levels in test samples from measured peak areas.

## Calibration Curve



**Figure 2.6:** Calibration curve of standard solutions of vitamin K

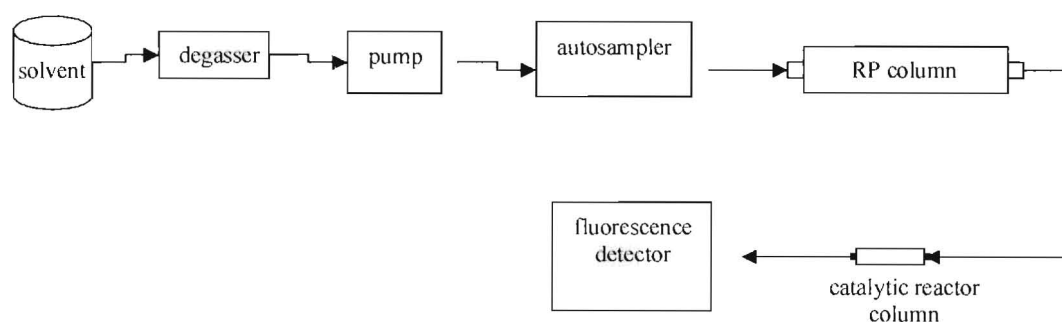
### 2.3 DEVELOPMENT OF AN HPLC ASSAY FOR VITAMIN K

Having investigated many aspects of the methanol/platinum black reduction system, this knowledge was then used for assay development. The focus of this study was the development of an HPLC assay for vitamin K levels in plasma, but this could also be applied to other biologically important quinones e.g. coenzyme Q. It was shown earlier

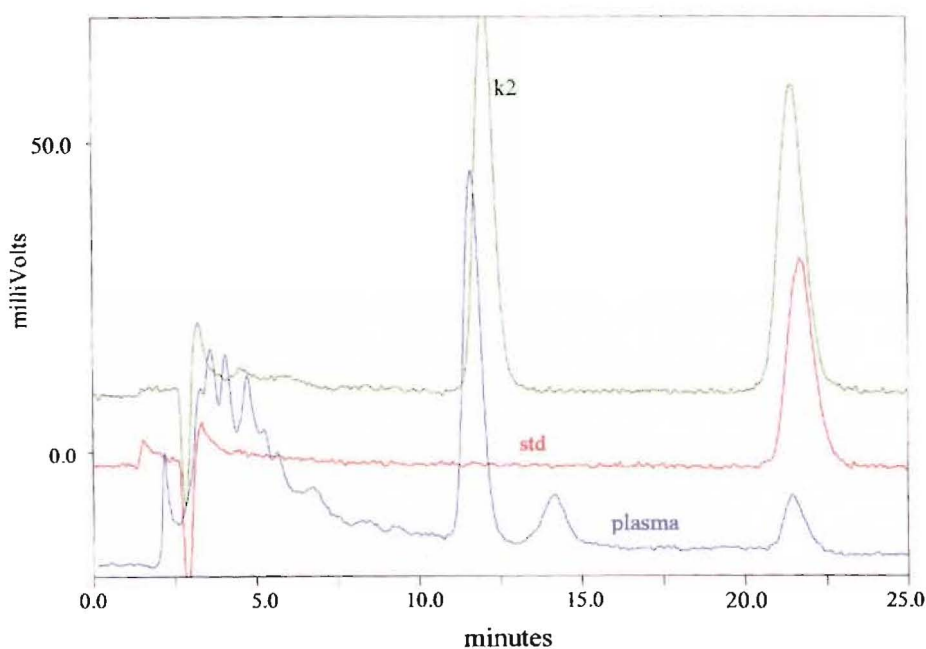
(see 4.2.3) that coenzyme Q was reduced by the platinum black/methanol system, although the increase in fluorescence was not as great as that seen for the K vitamins. It must be noted, however, that coenzyme Q is present in plasma at levels of approximately 1  $\mu$ M, which is several fold greater than the corresponding concentrations of vitamin K (0.4-1.5 nM). Due to the higher intrinsic levels of coenzyme Q in plasma, less sensitivity is required for accurate determination and quantitation.

The HPLC system used for the development of vitamin K assay consisted of a RP column, platinum black catalytic column and fluorescence detector (see figure 2.7). The mobile phase used was 100% methanol and the wavelengths for detection were excitation 249 nm and emission 408 nm. The sample extraction used for initial investigations was a standard procedure used for the extraction of other fat-soluble vitamins. This consisted of taking a plasma sample and adding an equal volume of ethanol to precipitate the proteins. To this hexane was added and the sample vortexed to extract the vitamin K into the organic phase. The hexane phase was removed, dried under a stream of nitrogen, and reconstituted into methanol. A sample of 50 $\mu$ L of this preparation was injected into the HPLC system described above and the chromatogram observed is shown in figure 2.8. Two major peaks were observed one with a retention time of 11.7 minutes and the expected peak corresponding to vitamin K<sub>1</sub> at a retention time of 21 minutes. It may be noted that these retention times may be decreased if necessary by the addition of isopropyl alcohol to the mobile phase.

Upon testing of other plasma samples the peak at 11.7 minutes was always observed, and the identity of this peak was of interest as it was well separated from contaminating lipids. A standard solution of MK-4 was prepared in ethanol at a concentration of 11 nM and run through the HPLC system. This was shown to have a retention time corresponding to the unknown peak observed in plasma preparations. This is of importance as this assay once validated may be used to simultaneously measure vitamin K<sub>1</sub> and MK-4 levels in plasma samples. Several groups have noted the presence of MK-4 in plasma samples, but as yet the biological function of this vitamer is unknown.



**Figure 2.7:** Schematic of HPLC system used for assay development.



**Figure 2.8:** HPLC chromatograms of a vitamin K<sub>1</sub> standard (red), a solution of K<sub>2</sub> and vitamin K<sub>1</sub> (green), and a pooled plasma sample (blue).

### 2.3.1 RECOVERIES

A series of spiked plasma samples were prepared by the addition of vitamin K<sub>1</sub> to pooled plasma, to give final concentrations of 5 nM, 10 nM, 20 nM, and 50 nM. Using the sample preparation described above the unspiked and 20 nM spiked plasma samples were prepared in duplicate. The results obtained from this experiment showed the

presence of vitamin K<sub>1</sub> in the unspiked plasma, and a strong fluorescence at 21 minutes in the 20 nM spiked plasma. To obtain a calibration curve and calculate the recovery of vitamin K from these spiked plasma samples this process was repeated with the five plasma samples each extracted in quadruplicate. Also run were standard solutions of vitamin K in methanol at concentrations of 10 nM and 50 nM. Results from this study were inconclusive. The peak areas obtained for the 20 nM spiked plasma were much smaller in this case, and no vitamin K<sub>1</sub> was detectable in the unspiked plasma. Variation was also obtained across the duplicate runs of each plasma concentration. To determine whether the plasma samples had deteriorated or the sample preparation varied, the 20 nM spiked plasma sample was reextracted and tested. The results obtained were comparable with the original findings, which implied the sample preparation was the cause of these inconsistencies. There are several possible reasons for this including variation in the extraction of vitamin K into the hexane phase and incomplete reconstitution of the vitamin K into methanol before injection. The reconstitution step is problematic as vitamin K is only slightly soluble in methanol, but other solvents are not compatible with the HPLC mobile phase. Possible solutions to this may include the addition of a small amount of a detergent to the reconstitution mixture or sonication to ensure all vitamin K is dissolved. The systematic development of a new sample preparation is an involved process, which falls beyond the scope of this project.

This study has investigated several aspects of the methanol/metal catalyst reduction system, as it may be applied to an HPLC assay system for several different quinones. This included an investigation of several different metal catalysts, and the effects of various mobile phases on the reduction process. For the purpose of this thesis we were particularly interested in the development of an assay for vitamin K and this was further studied. An assay system for the K vitamins, using post-column reduction with platinum black and methanol has been developed. This method has several advantages over the commonly used zinc metal reduction HPLC assay for vitamin K. The platinum black reactor lasts indefinitely removing the need for repacking of the catalyst column between each assay run, and the simple solvent system developed removes the need for zinc ions in the mobile phase, which can precipitate out within the HPLC system. The methanol mobile phase has been shown to effectively separate vitamin K from contaminating lipids in plasma samples. Studies have shown that the addition of isopropyl alcohol to the



mobile phase could be used to slightly reduce the retention times of components, thereby shortening the run time of the experiment. The HPLC assay method presented here is similar to that described by Usui *et al*<sup>30</sup> but the need for semi-preparative chromatography of plasma samples prior to analysis, to remove lipophilic contaminants has been removed. Upon the establishment of a consistent sample preparation method, this assay will be validated for routine use on patient samples.

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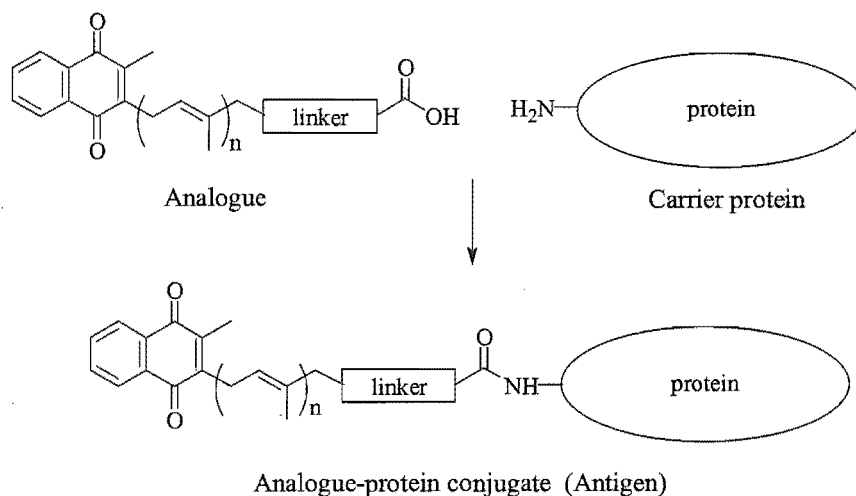
## **CHAPTER THREE**

### **SYNTHESIS OF VITAMIN K**

### **ANALOGUES**

### 3.1 INTRODUCTION

In addition to the development of a new HPLC assay for vitamin K, we were interested in investigating the feasibility of an immunoassay for vitamin K. The production of an enzyme-linked immunosorbent assay (ELISA) hinges on the successful preparation of monoclonal antibodies to the antigen of interest, in our case vitamin K (see chapter 4 for details). The first step in the production of antibodies is the inoculation of a host animal with a suitable analogue of vitamin K to act as an antigen (i.e. a foreign body which will evoke an immune response in the host). There are several factors that increase the chance of a strong immunogenic response in the test animal, and these include greater phylogenetic distance between the antigen and recipient, increased size of the antigen, repeating units within the molecule or aggregates of a molecule, and the use of adjuvants.<sup>1</sup> As vitamin K itself is a small molecule, which is naturally occurring in all mammals it cannot be used directly as the antigen for inoculation. Instead it is necessary to conjugate vitamin K to a carrier protein which will increase its size, immunogenicity, and solubility in aqueous media.

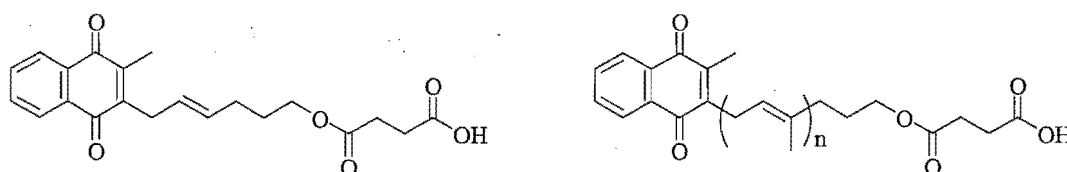


**Figure 3.1:** Schematic of conjugation of vitamin K analogue to carrier protein

To conjugate vitamin K to a carrier protein several analogues were designed, which contained a carboxylic acid moiety at the terminus of the isoprene side chain, while leaving the naphthoquinone head group unaltered. This approach was taken as the naphthoquinone ring is present in all forms of the vitamin and therefore, is the best point for antibody recognition. A carboxylic acid functionality was chosen to allow conjugation to the carrier protein via an amide bond to the amino group of the side

chain of lysine residues on the surface of the carrier protein (see figure 3.1). It is common, when conjugating a small molecule to a large protein, to use a 'linker' moiety between the small molecule and protein. This acts both as a spacer between the two components, so the small molecule protrudes from the surface of the protein and, as a convenient way of introducing the functionality required for conjugation.

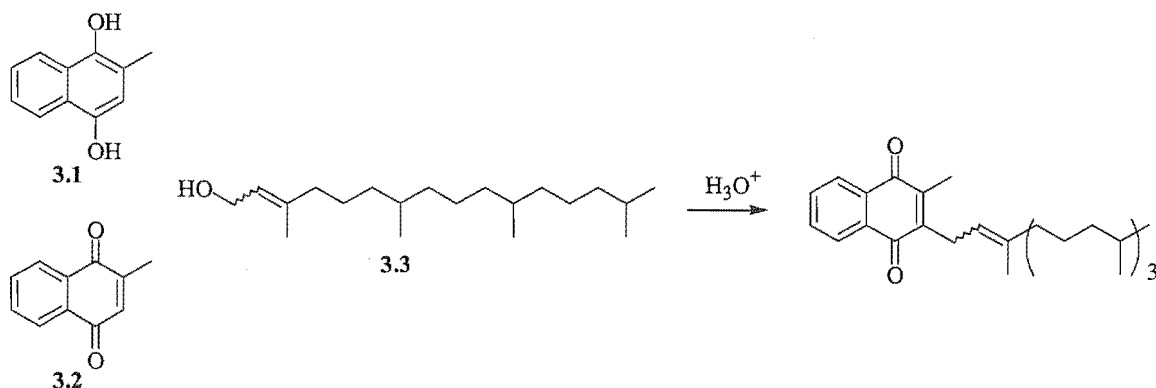
There are two possible approaches to the preparation of side-chain functionalised vitamin K analogues, the coupling of a core molecule to a functionalised side chain, or the synthesis of a polyprenyl K analogue followed by side chain functionalisation. This second approach calls for the investigation not only of previous syntheses of side-chain functionalised vitamin K analogues, but also of the synthesis of polyprenyl quinones in general. Discussed below are the previous synthetic approaches to vitamin K and side-chain functionalised vitamin K analogues. This is followed by a discussion of the synthesis of the target analogues shown in figure 3.2.



**Figure 3.2:** Target vitamin K analogues ( $n=1$  or  $3$ )

## 3.2 SYNTHESIS OF VITAMIN K

The first reported syntheses of vitamin K<sub>1</sub> came independently from the laboratories of Fieser<sup>2</sup>, Binkley *et al*<sup>3</sup> and Almquist and Klose<sup>4</sup> in 1939 by condensing 2-methyl-1,4-naphthohydroquinone or menadione (vitamin K<sub>3</sub>) and natural phytol in the presence of oxalic acid, and zinc dust in acetic acid, respectively (scheme 3.1).



**Scheme 3.1**

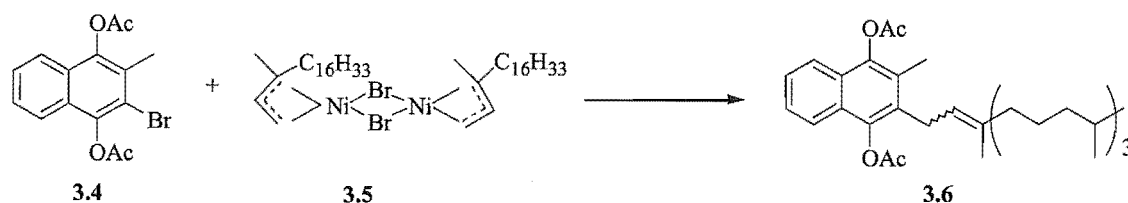
Although this gave the desired compound, yields were low and product purification was difficult. The major problems with this approach were chromanol formation (cyclic isomers of the corresponding hydroquinones), and the lack of control over the stereochemistry at the  $\Delta^2$  position of the polyprenyl side-chain, with the production of both the *cis* and *trans* isomers.

Since the early 1970's there has been increased interest in novel and efficient syntheses of vitamin K and related compounds, as the varied biological roles of the vitamins became apparent (see chapter 1). Most of these syntheses consist of the preparation of a protected core followed by the coupling of the desired side chain, and deprotection. There have been several syntheses of the varying protected core molecules for vitamin K with the choice mainly influenced by the deprotection procedure and the chemistry involved in the remainder of the synthetic pathway. Some of the more popular choices are 1,4-dimethoxynaphthalene, 1,4-diacetoxynaphthalene and quinone bisketals which can be deprotected by oxidation, or exposure to basic and acidic conditions respectively.

The key step in the synthesis of polyprenyl vitamin K analogues is the coupling of the isoprene side chain to the quinone nucleus. In early reports<sup>2-4</sup> this was carried out via Friedel-Crafts alkylation where the stereochemistry of the  $\Delta^2$  position of the side chain was of great interest. The desired product was that which possessed the all-*trans* configuration as this was shown to have higher physiological activity than the corresponding *cis* isomer. Under conventional Friedel-Crafts conditions the coupling of natural *trans*-phytol yielded, after oxidation, a quinone mixture containing approximately 90% *trans*-phyloquinone. An improvement on this method by Lindlar<sup>5</sup> using menadiol 1-benzoate as a starting material and  $\text{BF}_3$  etherate as the catalyst gave complete retention of stereochemistry of the side chain. This process avoids chromanol cyclization, and has been optimised to avoid 2-alkylation, but remains limited by the inherent instability of the allylic alcohol to the acidic conditions used.<sup>6</sup>

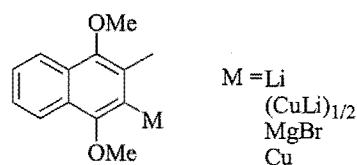
The coupling procedures used have advanced greatly since the introduction of transition metals into organic chemistry. The first reported use of transition metals in the synthesis of vitamin K was that of Sato *et al*<sup>7</sup> in 1973 who utilised allyl nickel complexes. The treatment of a *trans*-phytylnickel complex (3.5) with 2-bromo-3-methyl-1,4-diacetoxynaphthalene (3.4) afforded 3.6, which after deprotection and

silica chromatography, gave a mixture of the *cis*- and *trans*- vitamin K<sub>1</sub> (scheme 3.2). The ratio of these products was found to vary greatly with the solvent used and the best results were obtained when the reaction was carried out in *N*-methylpyrrolidone where vitamin K<sub>1</sub> was synthesised in 75% yield with a *cis:trans* ratio of 20:80. The synthesis of vitamin K<sub>2</sub> via an analogous route gave an 85% yield and 30:70 *cis:trans* ratio. The proportion of all-*trans* product was increased by low temperature recrystallisation but still only a 13:87 mixture was obtained.



**Scheme 3.2**

Just a year later Snyder and Rappaport reported the synthesis of a series of menaquinones via the formation of a 2-metallo-3-methyl-1,4-dimethoxynaphthalene and reaction of this with a suitable aldehyde or alkyl halide.<sup>8</sup> This approach varies from all earlier reports in that it is the naphthalene portion that is activated as a nucleophile in relation to the functionalised side chain. A series of different metallonaphthalenes (see figure 3.3), and coupling conditions, were investigated with Grignard coupling conditions proving the most reliable and giving good retention of *trans* stereochemistry (98-99%). Grignard coupling with allylic halides followed by oxidative deprotection to the quinone gave an 80% yield of MK-2 from geranyl bromide, and 73% overall yield of MK-9 from solanesyl bromide, with 2-bromo-3-methyl-1,4-dimethoxynaphthalene as a contaminating by-product.

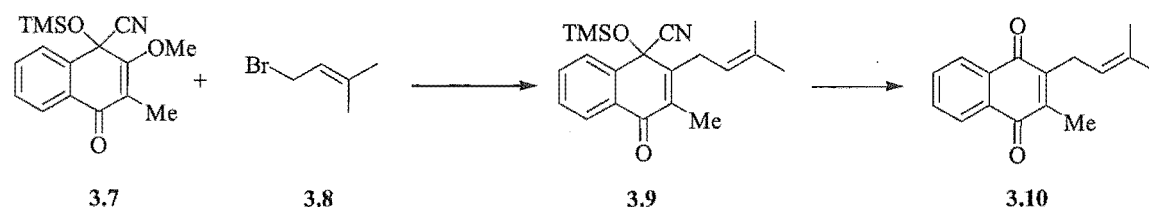


**Figure 3.3:** Metallonaphthalenes utilised by Snyder and Rappaport in the synthesis of MK's

In 1976 Evans and Hoffman published a method for regiospecific isoprenylation of benzo- and naphthoquinones (scheme 3.3).<sup>9</sup> Cyanide-catalysed addition of trimethylsilyl cyanide (TMSCN) to 2-methoxy-1,4-naphthoquinone gave the protected quinone 3.7. In situ Grignard addition of prenyl bromide to the protected quinone afforded the protected product 3.9, via 1,2-carbonyl addition followed by Cope rearrangement. Deprotection of the quinone with AgF gave MK-1 (3.10) in



71% overall yield.



**Scheme 3.3**

Various other conditions have also been used in the preparation of short side-chain K analogues including the use of  $\beta$ -cyclodextrin catalysis by Tabushi *et al.*<sup>10</sup> in 1979. This procedure was carried out in phosphate buffer containing 30% methanol, in the dark under an argon or nitrogen atmosphere, and was found to be very oxygen sensitive. After 9 hour reaction time yields ranged from 40-60%, with cyclodextrin thought to act as a ligase and/or oxygenase, i.e. a 'vitamin K synthase', in the reaction.

Chenard *et al.*<sup>11</sup> in 1980 again opted for activation of the quinone core in their report of a synthesis of the menaquinones and phyloquinone. Reaction of lithium organocuprates of quinone bisketals with allyl halides gave good stereoselectivity at the  $\Delta^2$  position and yields in the order of 90-95% based on halide. The cuprate formed in this reaction took the form  $R_2CuLi$ , and its reactivity towards coupling was found to be dependant on the nature of coupling substrate. This cuprate addition was found to be very versatile in the synthesis of this type of compound.<sup>11</sup> More than one R group was transferred when reaction was carried out with allyl bromide, while with benzoyl chloride and cyclohexylcarboxylic acid chloride one R group was transferred efficiently. Benzyl bromide was found to give good yields of the coupled product with one R group transferred, while methylbromoacetate afforded only low yields of a product which was difficult to purify.<sup>11</sup>

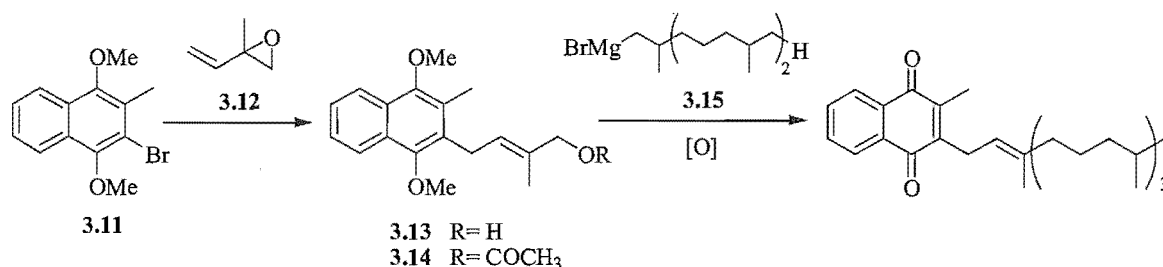
The same cuprate chemistry was also employed by Syper *et al.* who synthesised MK-1 and MK-2 from the reaction of 2-bromo-3-methyl-1,4-dimethoxynaphthalene with prenyl bromide and geranyl bromide respectively.<sup>12</sup> These coupling reactions proceeded in 68% and 78% yields, and were followed by oxidative deprotection of the quinone to give the K vitamins.

Maruyama *et al.*<sup>13</sup> in 1989 utilised allylstannanes to produce a series of benzo- and naphthoquinones photochemically. The reaction of menadione with allyltributylstannane in benzene or acetonitrile, with 315 nm light for 3 hours gave a mixture of products including 2-allyl-3-methyl-1,4-naphthoquinone in 15-40% yield.

Purification was achieved by recrystallisation where possible. One advantage over earlier syntheses was that no Lewis acid was required, as this was a thermal allylation.

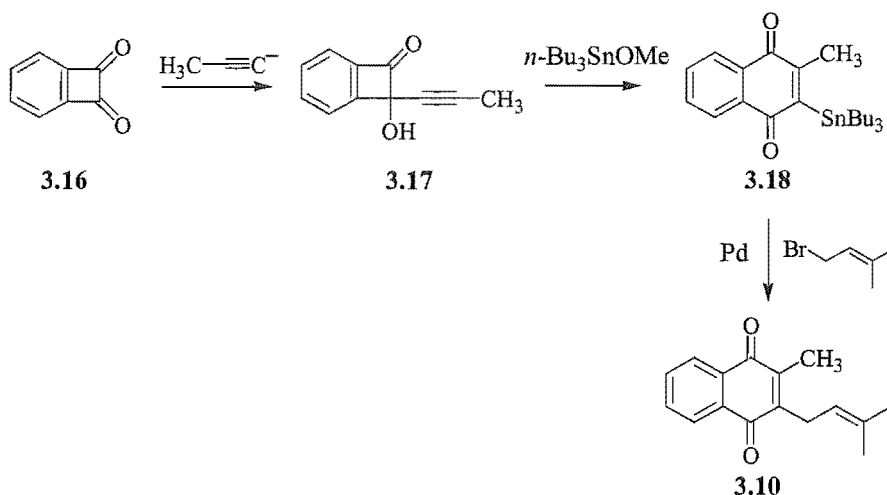
The use of lead bromide/aluminium powder as a catalyst for the prenylation of quinones has also been reported.<sup>14</sup> The advantage of this process over other known reagents is that it can be carried out at room temperature and gives only monoprenylated products. Also strictly anhydrous conditions are not required. Using this technique MK-1 was synthesised from 2-methyl-1,4-naphthoquinone in 50% yield.<sup>14</sup>

Another approach to the synthesis of the K vitamins is stepwise elongation of a short side chain analogue. Schmid *et al*<sup>15</sup> in 1990 published a synthesis of the 4 stereoisomers of phyloquinone using this strategy (scheme 3.4). Reaction of 2-bromo-3-methyl-1,4-dimethoxynaphthalene with isoprene epoxide via a Gringard reaction gave **3.13**. Side chain extension was then achieved by activation of the resulting alcohol as an ester (**3.14**), followed by reaction with an appropriate alkyl magnesiumbromide (**3.15**) to give the three unnatural isomers of vitamin K<sub>1</sub> with complete stereochemical retention. The natural isomer of phyloquinone was prepared by a novel synthesis involving O-alkylation of menadiol monoacetate with phytylchloride. A mild Lewis-acid catalysed rearrangement and deprotection of the quinone gave the desired phyloquinone. This is an improvement on earlier Lewis-acid based methods as it takes place in milder conditions with less catalyst required.



**Scheme 3.4**

Another novel approach reported by Liebeskind<sup>16</sup> was the synthesis of stannylquinones from alkynylcyclobutenones, and subsequent prenylation (scheme 3.5). Synthesis of 2-methyl-3-(tri-*n*-butylstannyl)-1,4-naphthoquinone (**3.18**) from the corresponding benzocyclobutenedione (**3.16**) proceeded in 49% yield over two steps. Subsequent palladium-catalysed cross coupling of this stannylquinone with prenyl bromide afforded MK-1 (**3.10**) in 90%.<sup>16</sup>



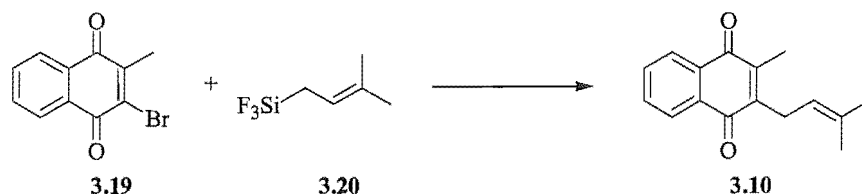
Scheme 3.5

Until the early 1990's a series of different allylmetallated compounds had been used in the synthesis of K vitamins and short chain analogues of these compounds. The use of Grignard or organolithium reagents required protection of the quinone to suppress quinone reduction, but allylzinc and  $\pi$ -allylnickel(II) reagents could be used for direct allylation without protection of the quinone nucleus.<sup>17</sup> Allylsilanes and allylstannanes had also been used for direct allylation of unprotected quinones in this way, but Lewis-acid catalysts were required owing to their low nucleophilicity. In 1991 Araki *et al*<sup>17</sup> utilised allylindium sesquihalides  $\text{R}_3\text{In}_2\text{X}_3$  ( $\text{R}=\text{allyl}$ ). In the synthesis of simple menaquinones only two of the three allyl groups were found to be transferred. Reaction of 2-bromo-3-methyl-1,4-naphthoquinones with prenylindium species gave rise to MK-1 in 67% yield.<sup>17</sup> In many cases studied, allylation occurred at a point of substitution on the quinone ring, but in this case reaction is thought to proceed via  $\gamma$ -prenylation at the least hindered carbonyl followed by [3,3] sigmatropic rearrangement to give exclusively MK-1.

Garcias *et al*<sup>18</sup> in 1994 produced menaquinone-4 by coupling 2-bromo-3-methyl-1,4-dimethoxynaphthalene with allylic aldehydes and  $n\text{BuLi}$  to give an alcohol product. Birch hydrogenolysis was then shown to proceed with complete retention of  $\Delta^2$  stereochemistry and when followed by oxidative deprotection of the quinone, gave MK-1 and MK-3 in 35 and 30% yield respectively.

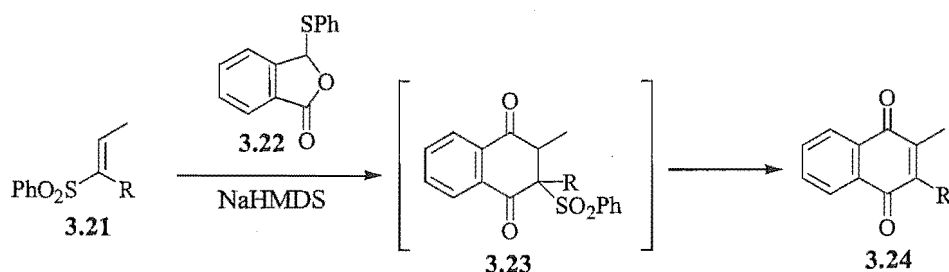
In 1995 Hagiwara *et al*<sup>19</sup> reported a synthesis of menaquinone-1 (MK-1) by the allylation of 2-bromo-3-methyl-1,4-naphthoquinone (3.19) with (3-methyl-2-butenyl)trifluorosilane (3.20) in formamide at 80 °C. This reaction occurred selectively at the carbon bearing the bromine (scheme 3.6). In contrast the same

reaction conditions using 2-methyl-1,4-naphthoquinone as the starting material saw reaction at the methylated 2-position. This was rationalised by the initial 1,2 addition of the silane taking place at the sterically less crowded carbonyl group and subsequent [3,3]-sigmatropic rearrangement to the observed products. This reaction was found to take place with high regioselectivity and MK-1 was formed in 54% yield after 68 hours reaction time.<sup>19</sup>



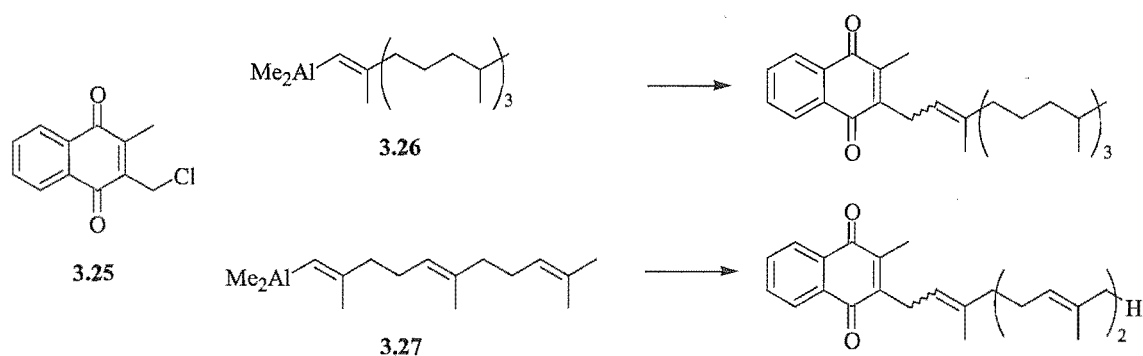
**Scheme 3.6**

In 1995 Tso *et al*<sup>20</sup> published a one-flask synthesis of the K vitamins (scheme 3.7). This involved an anionic [4+2] cycloaddition of 3-substituted isobenzofuranones (3.22) to alkenylsulfones (3.21). The sulfones were prepared in one flask from allyl phenyl sulfone, used as initial Michael acceptors and, after cyclization the sulfonyl group was eliminated *in situ* under the reaction conditions. This produced phylloquinone in 61% yield and MK-1, 2 and 9 in 60, 63 and 64% yield respectively.<sup>20</sup>



**Scheme 3.7**

More recently, Lipshutz utilised Ni(0) catalysis in the synthesis of phylloquinone and menaquinones (scheme 3.8).<sup>21</sup> A chloromethylated quinone core (3.25) was prepared either by direct chloromethylation of the quinone, or from oxidation of corresponding benzylic chlorides. This core was reacted with a trimethylaluminium functionalised side-chain (3.26 or 3.27), prepared by carbo-alumination of the corresponding terminal alkyne, in the presence of 0.5 mol% Ni(0) catalyst to give racemic phylloquinone in 88% yield and MK-3 in 93% yield.<sup>21</sup>



Scheme 3.8

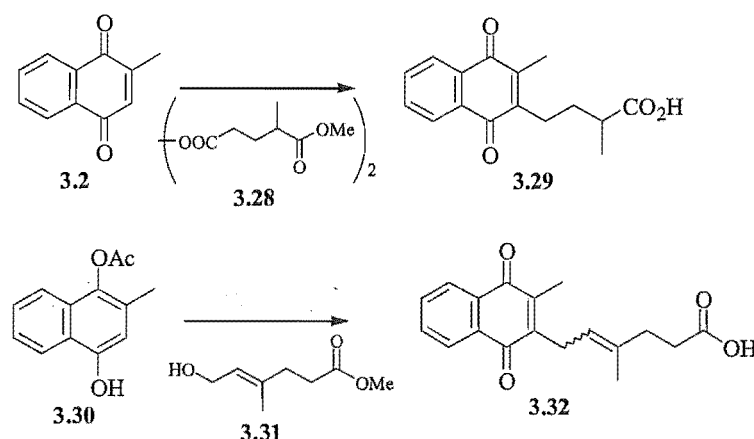
### 3.3 PREVIOUS WORK ON FUNCTIONALISED ANALOGUES OF VITAMIN K

As well as there being significant interest in the efficient and stereoselective synthesis of vitamin K, over the same period there has also been interest in the synthesis of functionalised analogues of the K vitamins. These compounds have been synthesised for a variety of reasons from the production of inhibitors of vitamin K dependant carboxylase and vitamin K epoxide reductase,<sup>22,23</sup> to an investigation of metabolites of the K vitamins. A wide variety of analogues have been synthesised including substituted vitamin K epoxides,<sup>22,23</sup> methylated naphthoquinones<sup>24</sup> and side-chain functionalised vitamin K analogues. It is the side-chain functionalised analogues which are of particular interest in this work and these will form the focus of this thesis. In the last three decades there have been several attempts to synthesise such compounds.<sup>25-32</sup>

From 1974 Okamoto and Watanabe published a series of papers looking at the synthesis of metabolites of phylloquinone, ubiquinone and  $\alpha$ -tocopherol, in order to study the structure activity relationships of these compounds.<sup>26</sup> The isolation of urinary metabolites of these quinones and the possibility that these metabolites may be the active forms *in vivo* lead to this work.

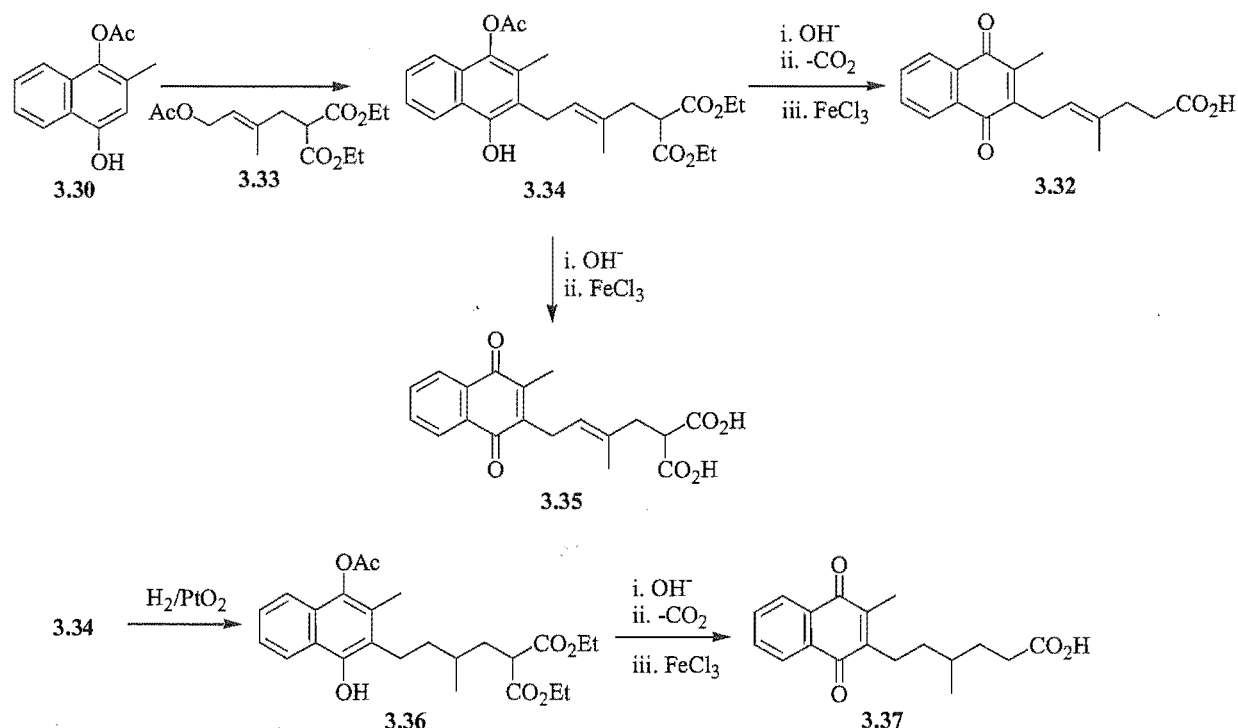
The earliest compounds published were 3.29 and 3.32 in 1974.<sup>27</sup> Reaction of 2-methyl-1,4-naphthoquinone (3.2) with  $\gamma,\gamma'$ -dimethoxycarbonylvaleryl peroxide (3.28) in acetic acid, followed by ester hydrolysis gave 3.29 in 50% yield from the peroxide side chain. However, this peroxide side chain was prepared via a three step synthesis in only 5% yield, making this approach less attractive. Condensation of 1-acetoxy-4-hydroxy-2-methylnaphthalene (3.30) with methyl 6-hydroxy-4-methylhex-4-enoate

(**3.31**) gave a 3:1 mixture of *cis:trans* isomers of **3.32**. An attempt to synthesis purely the *trans* isomer of **3.32** by using the *trans*-allylic alcohol was unsuccessful as *cis/trans* isomerisation occurred during condensation.<sup>27</sup> Compound **3.32** is a possible target for our study but there are several problems with this synthesis. As above, the side chain used (**3.31**) must be synthesised via a multi-step synthesis with only moderate yield. Also *cis/trans* isomerisation occurs during the coupling step, which means the overall yield of the desired *trans* isomer for the coupling is less than 20%, and requires rigorous purification via recrystallisation.

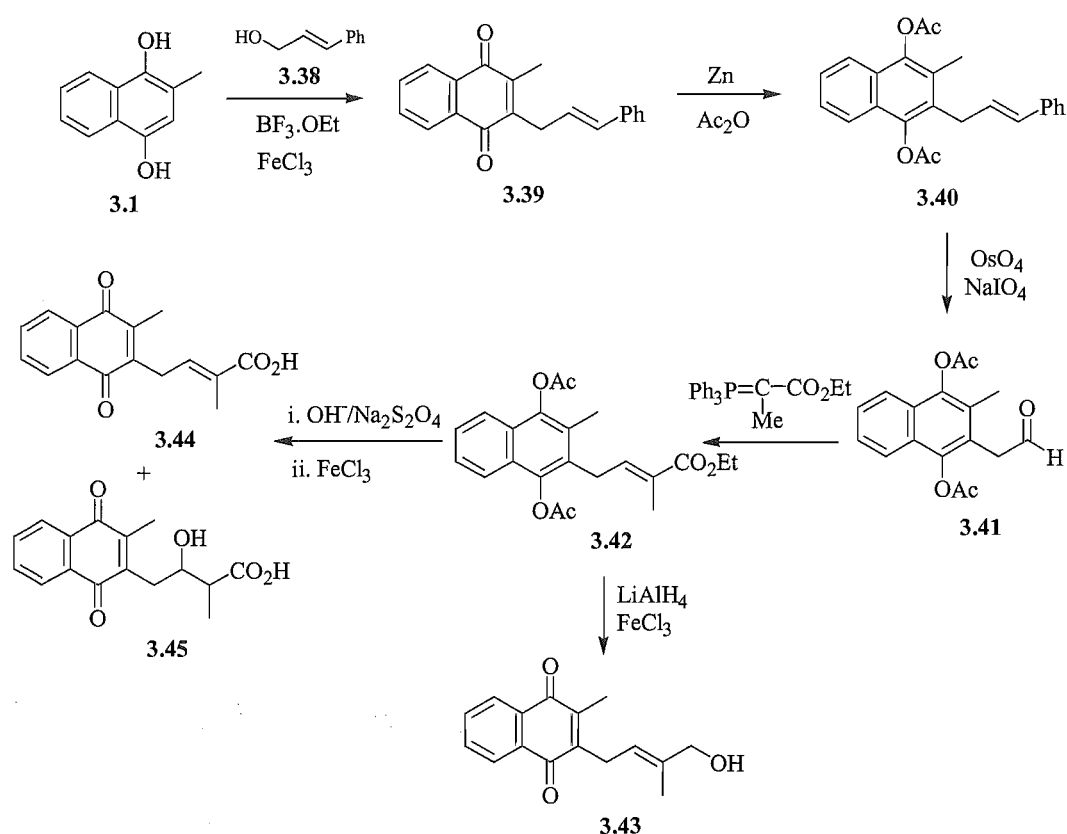


**Scheme 3.9**

In 1978 the synthesis of a series of carboxylated analogues was reported.<sup>28</sup> The condensation of 1-acetoxy-4-hydroxy-2-methyl-naphthalene (**3.30**) with *trans*-diethyl-4-acetoxy-2-methyl-2-butenylmalonate (**3.33**) gave intermediate **3.34** which was hydrolysed and oxidised to the diacid **3.35** (see scheme 3.10). Although the  $\text{BF}_3$ -etherate coupling used in the synthesis of **3.34** proceeded in 66% yield, the side chain moiety (**3.33**) is not readily available, and must be synthesised in four steps from isoprene. Intermediate **3.34** was also hydrolysed, decarboxylated and oxidised to give the acid **3.32** in 33% yield. This is an alternative synthesis of **3.32**, to that described earlier by Watanabe *et al.*,<sup>27</sup> but it still requires use of a side chain moiety which is only available via a multi-step synthesis. Catalytic hydrogenation of **3.34** followed by hydrolysis, decarboxylation and oxidation to the quinone yielded the 2',3'-dihydro derivative **3.37**.

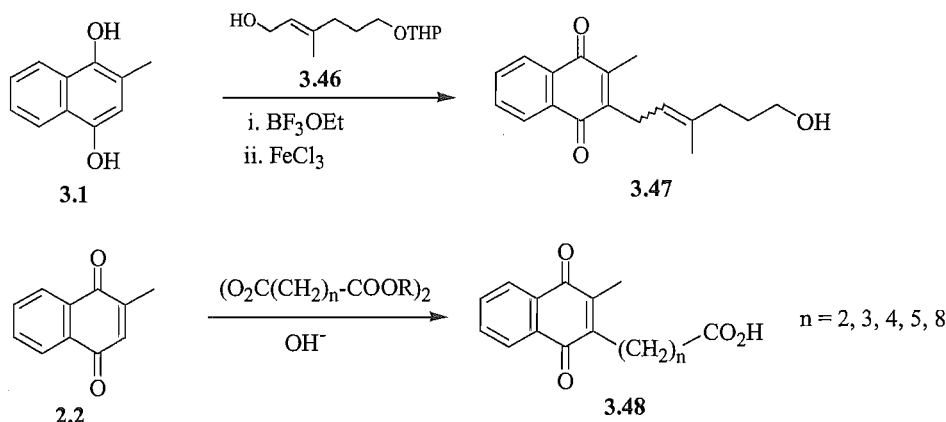
**Scheme 3.10**

Finally, in 1982 the synthesis of a series of vitamin K analogues bearing an alcohol functionality was published (scheme 3.11).<sup>26</sup> Condensation of 2-methyl-1,4-naphthaquinol (**3.1**) with cinnamoyl alcohol (**3.38**) followed by oxidation with ferric chloride afforded phenylpropenylquinone **3.39**. Reductive acetylation gave the acetyl protected quinone **3.40** and cleavage of the alkene with osmium tetroxide and sodium periodate gave the corresponding aldehyde (**3.41**). The Wittig reaction was used to extend the side chain giving ester **3.42**. Reaction with base and oxidation with ferric chloride afforded the acid functionalised analogue **3.44**, and a secondary alcohol side-product **3.45**. Reduction of the ester **3.42** with lithium aluminium hydride afforded the desired alcohol **3.43**. Both the alcohol (**3.43**) and the acid (**3.44**) presented here are possible targets for our study. Although the side-chain moiety in this procedure is the readily available cinnamoyl alcohol (**3.38**), the  $\text{BF}_3$ -etherate coupling proceeds in only 28% yield. This, together with a yield of only 24% for the final deprotection/oxidation step, gives an overall yield of **3.44** of only 4%. Similarly, the reduction of **3.42**, and subsequent oxidation to give the alcohol (**3.44**) proceeds in 62% yield, to give an overall yield of 10%.<sup>26</sup>

**Scheme 3.11**

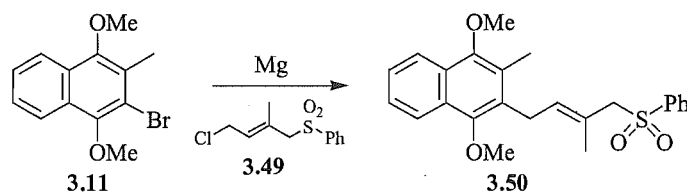
In a related sequence the Friedel-Crafts reaction of protected side chain 3.46 with 2-methyl-1,4-naphthoquinone (3.1) and subsequent oxidation with ferric chloride afforded a *cis/trans* mixture of alcohol 3.47 in 29% yield (scheme 3.12). The mixture of isomers is introduced here by the use of a *cis/trans* mixture of 3.46. As the target for our study is the production of a pure sample of the corresponding *trans* isomer, this route was not considered further. Alkyl alcohol analogues (3.48) were also obtained by the reaction of 2-methyl-1,4-naphthoquinone (3.1) with diacylperoxides, followed by hydrolysis of the ester group with base.





Scheme 3.12

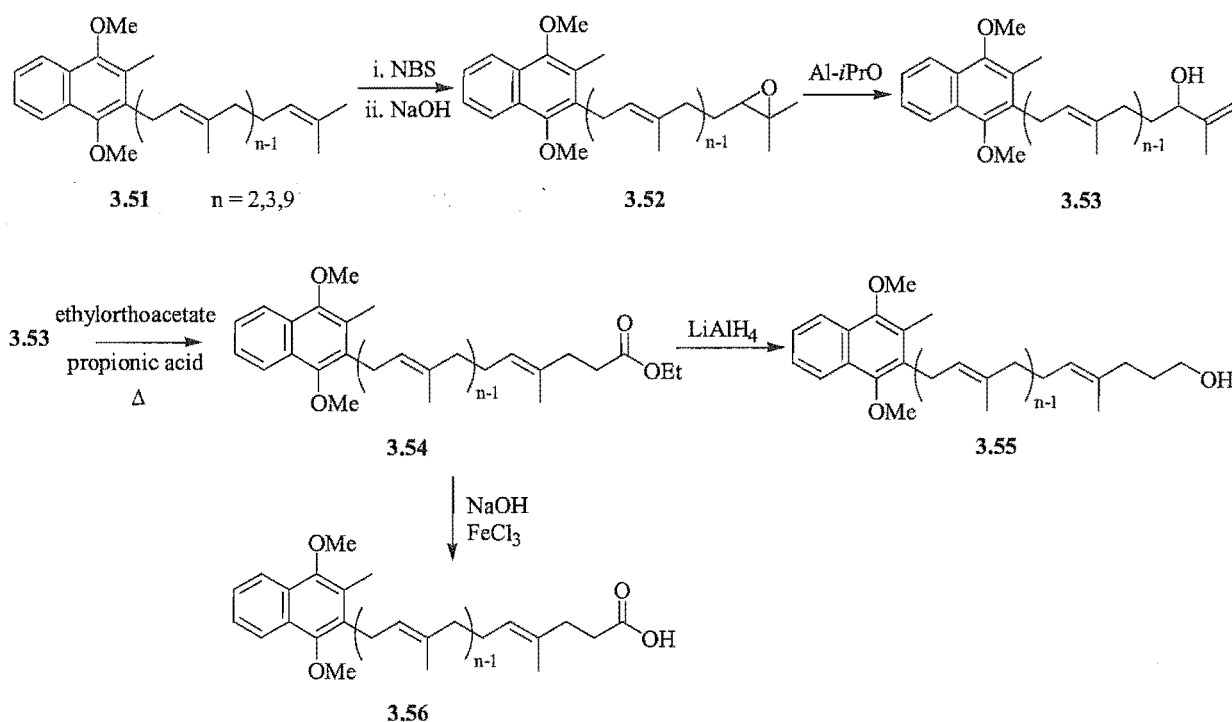
An efficient synthesis of sulfone functionalised analogues of vitamin K with a protected quinone core was published by Fujita *et al* (scheme 3.13).<sup>29</sup> This was achieved via the Grignard coupling of 2-bromo-3-methyl-1,4-dimethoxynaphthalene (3.11) with *trans*-4-chloro-2-methyl-1-phenylsulfonyl-2-butene (3.49), which was conveniently obtained from the reaction of isoprene with benzenesulfonyl chloride in the presence of copper chloride/TEA hydrochloride as catalyst. This reaction proceeded cleanly to give the desired product (3.50) in 82% yield.



Scheme 3.13

In 1982 Terao *et al*<sup>30</sup> published the synthesis of a series of quinone derivatives with modified polyprenyl side chains (scheme 3.14) and investigated the inhibitory effects of these compounds on the slow reacting substance of anaphylaxis (SRS-A). The regio-specific modification of the terminal prenyl group of the ubiquinone, menaquinone or tocoquinone side chain gave a convenient synthesis of quinone acids, quinone amides, quinone alcohols and quinone methylketones. These compounds were arrived at via side chain functional group interconversions of parent quinones with various polyprenyl side chains, leading to a library of related compounds for assay purposes. The two routes used to obtain these compounds rely on the Claisen rearrangement and the Carrol reaction. The carboxylic acid (3.56) synthesised here is an attractive target for our study. In this synthesis isomerisation of the epoxide (3.52)

to the allylic alcohol (3.53) and Claisen rearrangement to the ester (3.54), was followed by deprotection and oxidation to give the acid (3.56). This series of steps increases the length of the side-chain moiety by two carbon units as well as introducing the carboxylic acid functionality. As the yields for many of these reactions are not reported it is impossible to assess the overall efficiency of this synthesis. As the overall length of the side-chain is not important for our study, it was recognised that the epoxide could be directly cleaved to the corresponding aldehyde, removing a step from the synthesis described here. This aldehyde may then be reduced to the alcohol to allow addition of a 'linker' (scheme 3.26), or oxidised to the carboxylic acid (see section 3.4).



### Scheme 3.14

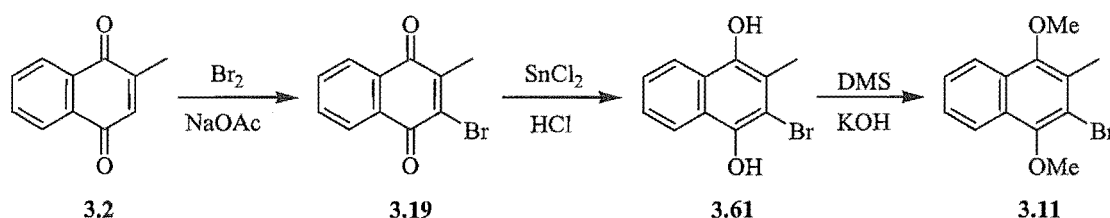
In an investigation of the alkylation of quinones by carbanions, and the mechanism of this reaction, Aldersley *et al* published a synthesis of a series of side-chain esters of 1,4 naphthoquinones.<sup>31</sup> The reaction of pyridinium salts with 2-methyl-1,4-naphthoquinone in the presence of triethylamine, or the reaction of the pyridinium salt and 2-methyl-1,4-naphthoquinone with potassium carbonate and a catalytic amount of 18-crown-6 ether were the approaches taken. Yields of products varied with the method and pyridinium salt used between 55 and 85%.<sup>31</sup>

In 1990 Barton *et al* were investigating the synthesis of hindered quinones via



### 3.4.1 SYNTHESIS OF NAPHTHALENE CORE

Of the many different quinones or protected quinones used in the synthesis of vitamin K molecules it was decided to use 2-bromo-3-methyl-1,4-naphthalene (3.11) for this study. This particular compound was chosen as it was stable to all chemical conditions used in the preparation of vitamin K analogues, but could be readily deprotected to reveal the corresponding quinone under oxidative conditions. The method chosen for the synthesis of 3.11 was that reported by Adams *et al*<sup>33</sup> outlined in scheme 3.15.



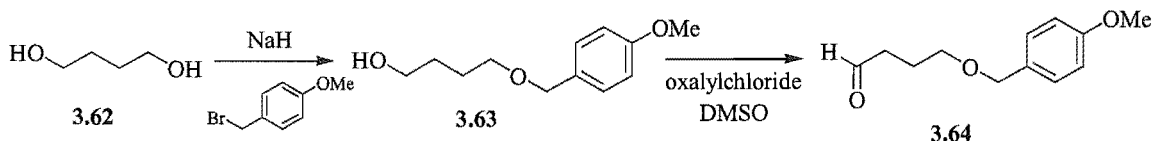
**Scheme 3.15**

The bromination of menadione (3.2) was achieved by reaction with bromine in the presence of glacial acetic acid and anhydrous sodium acetate with protection from light, and yielded 2-bromo-3-methyl-1,4-naphthoquinone (3.19) in 71% yield after recrystallisation. Reduction of quinone 3.19 to the hydroquinone 3.61 was achieved in 93% yield via reaction with stannous chloride in conc. hydrochloric acid. Although this hydroquinone (3.61) could be stored at -4 °C for some time, it was found to undergo a slow reoxidation to the quinone 3.19 even at low temperature. However, portionwise addition of an aqueous solution of potassium hydroxide to a suspension of 2-bromo-3-methyl-1,4-dihydroxynaphthalene (3.61) in dimethylsulfate (DMS), followed by ether extraction afforded the stable 2-bromo-3-methyl-1,4-dimethoxynaphthalene (3.11) as pale orange crystals, with melting point of 83-84 °C, in 64% yield.

Using the method of Adams *et al*,<sup>33</sup> 2-bromo-3-methyl-1,4-dimethoxynaphthalene (3.11) was successfully synthesised from vitamin K<sub>3</sub> (3.2) in 42% yield over all steps.

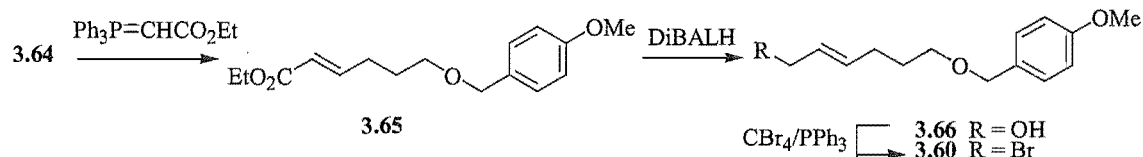
### 3.4.2 SYNTHESIS OF FUNCTIONALISED SIDE-CHAIN

The synthesis of the required functionalised side-chain (**3.60**) was achieved from the readily available 1,4-butanediol (scheme 3.16). Monoprotection of 1,4-butanediol with *p*-methoxybenzyl bromide<sup>34</sup> gave **3.63** in 48% yield, following column chromatography. Swern oxidation of the alcohol **3.63** to the aldehyde **3.64** proceeded in 83% yield.



**Scheme 3.16**

The  $\Delta^2$  double bond was introduced by reacting aldehyde **3.64** with [(ethyl-carbonyl)methylene]triphenylphosphorane in dichloromethane to give the allylic ester **3.65** in 80% yield (scheme 3.17). Reduction of ester **3.65** with diisobutyl-aluminium hydride afforded the allylic alcohol **3.66** as a yellow oil in 80% yield. The portionwise addition of triphenylphosphine to a solution of allylic alcohol **3.66** and carbon tetrabromide gave the allylic bromide **3.60** in 77% yield, after radial chromatography.

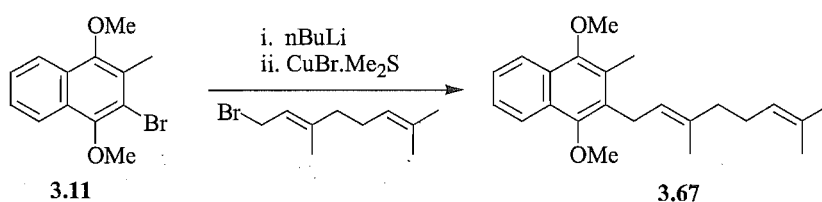


**Scheme 3.17**

A new synthetic route to the allylic bromide **3.60** is presented. The synthesis of the aldehyde **3.64** has been previously reported by Onoda *et al.*,<sup>34</sup> and we have used this as a precursor for the Wittig reaction to produce the allylic ester **3.65**. Reduction and bromination of this afforded the desired allylic bromide **3.60**. This allylic bromide has been previously reported by Hayashi *et al.*,<sup>35</sup> where synthesis proceeded from the allylic alcohol **3.66**, which in turn was produced via reduction of the corresponding alkyne.

### 3.4.3 COUPLING REACTION

Although there have been many different methods used for the coupling of an allylic halide to an aryl core the one chosen for this work was a copper mediated coupling. Syper *et al.*<sup>12</sup> had successfully used this method in the synthesis of similar compounds (see section 3.2) without observation of any isomerisation of the side chain. As the side chain (**3.60**) was produced via a multi-step synthesis a model system, geranyl bromide and 2-bromo-3-methyl-1,4-dimethoxynaphthalene, which has previously been reported, was attempted initially (scheme 3.18).<sup>12</sup> This reaction was found to proceed smoothly providing all solvents were freshly distilled and every precaution was taken to exclude water from the system.



**Scheme 3.18**

Reaction of **3.11** with nBuLi at 0 °C for 30 minutes was followed by cuprate formation via the addition of copper bromide dimethylsulfide complex. Addition of geranyl bromide to the reaction, followed by stirring for five hours and quenching with HCl afforded a crude product mixture. Purification by radial chromatography afforded three fractions, which upon <sup>1</sup>H NMR analysis were shown to contain 2-methyl-1,4-dimethoxynaphthalene, unreacted geranyl bromide, and the desired geranyl naphthalene (**3.67**) in 80% yield. This yield compares favourably with that of 78% reported by Syper *et al.* for the same reaction.<sup>12</sup> No isomerisation of the Δ<sup>2</sup> double bond was evident by <sup>1</sup>H NMR spectroscopy, but trace amounts of the corresponding terminal alkene, produced by attack at the 3-position of the allylic bromide, were observed. Distinctive multiplets at 4.92 and 6.35 ppm in the <sup>1</sup>H NMR spectrum, corresponding to the allylic protons, identified this terminal alkene.

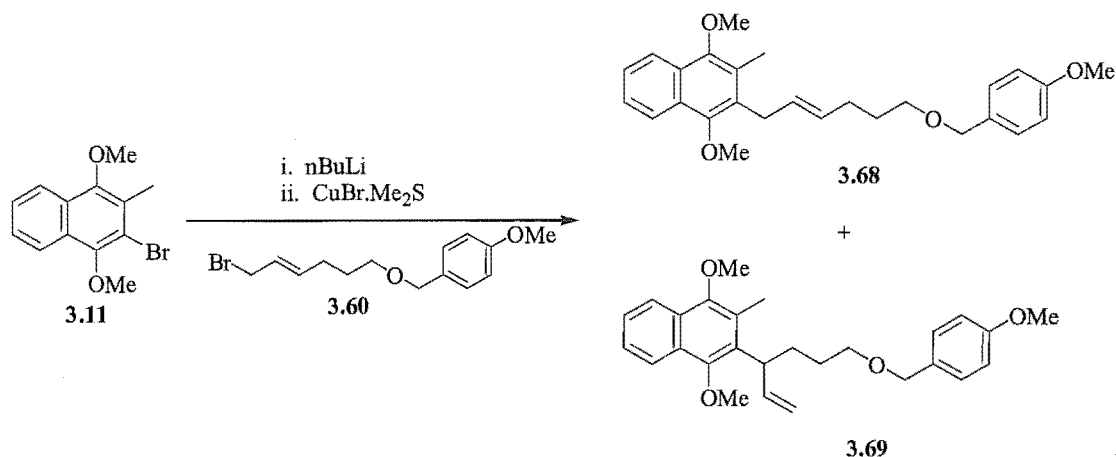
This reaction is thought to proceed via a diaryl cuprate, or Gilman's cuprate, which is prepared by the reaction of an organolithium with CuX (X= I, Cl, Br). Use of CuBr in its uncomplexed state is rare, and often leads to the formation of by-products due to the presence of Cu(II).<sup>36</sup> As the dimethylsulfide complex, however, it is an excellent cuprate precursor, as it is ether soluble and readily prepared free of

Cu(II).<sup>37</sup> Copper bromide dimethylsulfide complex must, however, be used fresh or purified by recrystallisation and stored under argon, as it readily loses dimethylsulfide which disrupts the stoichiometry of the reaction.<sup>37</sup> It is also important to know precisely the concentration of the organolithium used by titrating the *n*BuLi before use. Once formed the Gilman's cuprate can be reacted with almost any electrophile, with the most efficient being primary centres bearing iodide, bromide or tosylate.<sup>37</sup>

It is usual for one of the aryl groups of the Gilman's cuprate to be transferred during reaction with the substrate, and for this reason often two equivalents of the cuprate are reacted with one equivalent of substrate.<sup>11</sup> However, in the case of some substrates more than one aryl group is observed to be transferred. An investigation of the effect of substrate on the transfer of the aryl group by Chenard *et al* showed that allylic bromide gave greater than one R group transferred while benzoyl chloride and cyclohexylcarboxylic acid showed the transfer of only one R group.<sup>11</sup> Benzyl bromide gave moderate yields with less than one group transferred while methylbromoacetate gave very low yields and lead to a crude product mixture which proved difficult to purify.<sup>11</sup> The results observed from our model system are consistent with these findings, with geranyl bromide giving a yield of 80% of the coupled product with respect to the quinone core, showing more than one R group per Gilman's cuprate was transferred.

With the coupling process established the coupling of the prepared functionalised side chain (**3.60**) with 2-bromo-3-methyl-1,4-dimethoxynaphthalene under the same conditions was undertaken (scheme 3.19). Work-up and purification by radial chromatography afforded three fractions. Analysis of these fractions identified the desired product (**3.68**) in fraction three, which still contained a mixture of compounds. The desired product (**3.68**) was identified by the presence of several distinctive proton NMR resonances. A doublet at 3.55 ppm is characteristic of the benzylic proton resonance and is the most diagnostic signal to determine that coupling has occurred. Also present in the spectra are two multiplets at 5.35 and 5.61 ppm which were assigned to the allylic protons of **3.68**, and appear as two distinct signals in contrast to the corresponding proton resonances in **3.60** which occur as a single multiplet at 5.67 ppm. Another compound in the product mixture was the corresponding terminal alkene (**3.69**), which arises through attack of **3.60** at the C-3 rather than the C-1

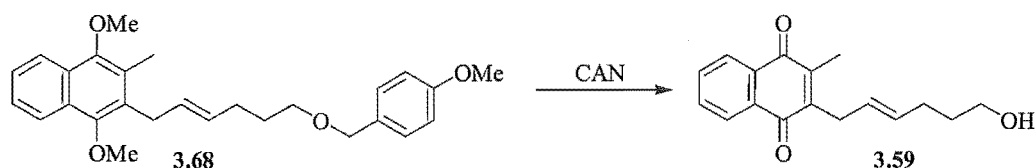
position. The characteristic splitting patterns of the  $^1\text{H}$  NMR signals at 5.02 and 6.23 ppm support the presence of a terminal alkene as part of the product mixture. All efforts to separate these compounds proved unsuccessful, so the product mixture was used in subsequent synthetic steps without further purification.



**Scheme 3.19**

Oxidative deprotection of the quinone core using ceric ammonium nitrate (CAN) was expected to also remove the *p*-methoxybenzyl protecting group of the side chain affording the alcohol (**3.59**) (scheme 3.20). Addition of a solution of CAN to the product mixture containing **3.68** afforded a crude product, which  $^1\text{H}$  NMR analysis showed to be a complex mixture of compounds. Purification by radial chromatography afforded two fractions, which were analysed by  $^1\text{H}$  NMR. Although a molecular ion at 270.12 was observed in fraction 1, which is consistent with the desired product (**3.59**), by  $^1\text{H}$  NMR analysis this fraction still contained several compounds. Due to a series of repeating signals, i.e. two doublets appearing at 3.35 and 3.44 ppm and two triplets appearing at 3.61 and 3.70 ppm, it was postulated that the isolated fraction may contain a mixture of the *cis* and *trans* isomers of **3.59**, although no evidence of a mixture of isomers was observed at earlier stages of the synthesis. It is impossible to establish any differences in the allylic protons of these compounds as a single broad multiplet is observed at 5.50 ppm for all the present allylic protons. This fraction also showed traces of *p*-methoxy benzyl alcohol and *p*-methoxy benzaldehyde by  $^1\text{H}$  NMR analysis.





Scheme 3.20

In summary, the known cuprate coupling reaction of 3.11 with geranyl bromide, was used as a model system for the coupling of the functionalised side chain. Using the reaction conditions described by Syper *et al.*<sup>12</sup> both of these reactions were successfully completed. In each case the crude product contained a mixture of 2-methyl-1,4-dimethoxynaphthalene, the desired product and the corresponding terminal alkene, which were separable by radial chromatography. Due to difficulties in the separation of the product mixture obtained from the oxidative deprotection of 3.68, it was decided to pursue a different approach to the synthesis of functionalised analogues of vitamin K.

### 3.5 ANALOGUE SYNTHESIS VIA SIDE CHAIN FUNCTIONALISATION

Due to the problems encountered during the final stages of the synthesis of analogue 3.59 it was decided to revisit the model coupling described above, and utilise the product (3.67) to obtain an appropriate analogue of vitamin K. This was achieved via functionalisation of the  $\Delta^6$  position of the side chain of 3.67. There were certain advantages to this approach as it maintains the *trans* stereochemistry at the  $\Delta^2$  position, and the methyl substitution at the 3 position of the side chain. This approach is similar to that taken by Terao *et al.*<sup>30</sup> and led to the design of a new target analogue (3.70) (figure 3.5), which incorporates a succinic acid linker moiety for conjugation of the analogue to a carrier protein.

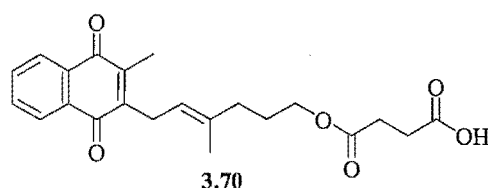
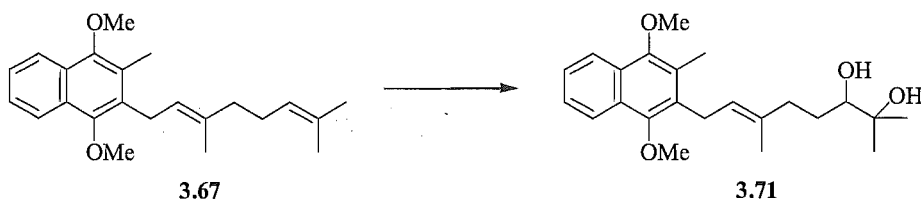


Figure 3.5: Structure of target analogue 3.70

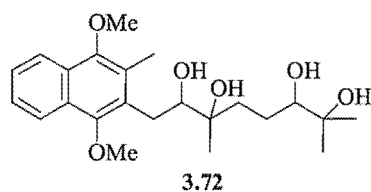
The initial functionalisation of the  $\Delta^6$  double bond of 3.67 is possible via dihydroxylation or epoxidation of this alkene. In the synthesis of juvenile hormone

III Crispino and Sharpless showed that the osmium catalysed asymmetric dihydroxylation of methyl farnesoate in the presence of the phthalazine ligand (DHQD)<sub>2</sub>-PHAL, gave a 20:1 ratio of products with dihydroxylation occurring at the  $\Delta^{10}$  to  $\Delta^6$  positions respectively.<sup>38</sup> This selectivity for the terminal, least hindered alkene is analogous to that required in the functionalisation of the geranyl side chain of **3.67** (scheme 3.21). Accordingly, the method developed by Crispino *et al* was applied to **3.67**.<sup>38</sup> Reaction of **3.67** and a solution containing (DHQD)<sub>2</sub>PHAL, potassium ferricyanide, potassium carbonate, methane sulfonamide and osmium tetroxide in *t*-butanol:water for 24 hours at 4 °C was followed by dichloromethane extraction. <sup>1</sup>H NMR analysis showed only starting material (**3.67**) was present in the reaction mixture. It is possible that the phthalazine ligand is hindering this reaction by co-ordinating to the naphthalene ring of **3.67**.



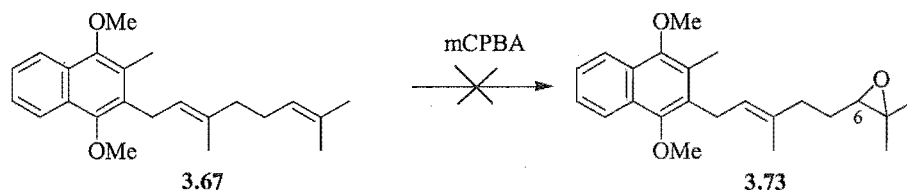
**Scheme 3.21**

As the stereochemistry of the dihydroxylation will be lost during cleavage to the corresponding aldehyde, and therefore asymmetric dihydroxylation is not required, other methods for dihydroxylation of an alkene were investigated. One common method for dihydroxylation is reaction with *N*-methylmorpholine-*N*-oxide in the presence of osmium tetroxide or potassium osmate.<sup>39</sup> A solution of **3.67** in acetone was added to a solution containing potassium osmate and *N*-methylmorpholine-*N*-oxide in a combined acetone:*t*-butanol:water solvent. After 24 hours the reaction was quenched and crude product isolated. This was purified by radial chromatography to give four fractions, which were analysed by <sup>1</sup>H NMR spectroscopy. Fraction one was found to contain 2-methyl-1,4-dimethoxynaphthalene, while fraction two contained only a mixture of minor by-products. The desired diol (**3.71**) was isolated in fraction three, and obtained in 30% yield, while fraction 4 was tentatively assigned as contained the corresponding tetraol (**3.72**) in 20% yield (see figure 3.6). The <sup>1</sup>H NMR spectrum of the tetraol (**3.72**) showed complex splitting, indicative of the presence of a mixture of diastereoisomers.



**Figure 3.6:** Structure of tetraol by-product from dihydroxylation of **3.67**

As the yield of diol **3.71** was low and purification difficult, epoxidation of the alkene was investigated as another possible route to the introduction of functionality into the isoprenoid side-chain of **3.67**. Reaction of **3.67** with *m*-chloroperbenzoic acid in dichloromethane for 2 hours at 0 °C showed the absence of starting material by TLC analysis. Excess *m*-chloroperbenzoic acid was removed by the addition of sodium metabisulfite and a crude reaction product isolated. Purification by radial chromatography afforded two fractions. The first of these fractions contained predominantly starting material, while the second, although showing a triplet at 2.67 ppm, consistent with H-6 of the desired product (**3.73**), showed no resonance for the allylic proton at 5.17 ppm. As neither of these fractions contained the desired epoxide (**3.73**), this method was not further investigated.

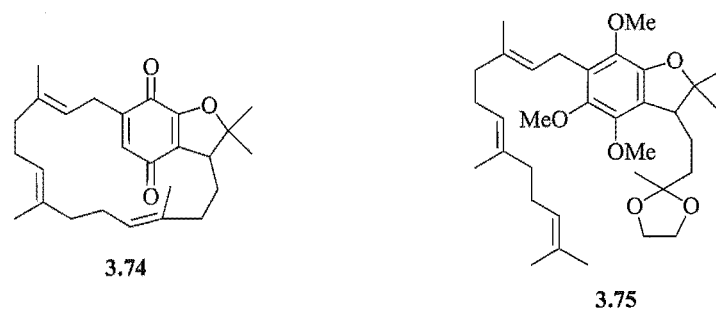


**Scheme 3.22**

Given the problems encountered with the above epoxidation process it was decided to attempt reaction of **3.67** with sodium acetate and peracetic acid at -10 °C. All literature procedures require the use of 40% peracetic acid, but only 20% peracetic acid was available. The crude product obtained from this reaction was analysed by  $^1\text{H}$  NMR and, although some of the desired product (**3.73**) was present, purification by silica chromatography afforded **3.73** in less than 5% yield.

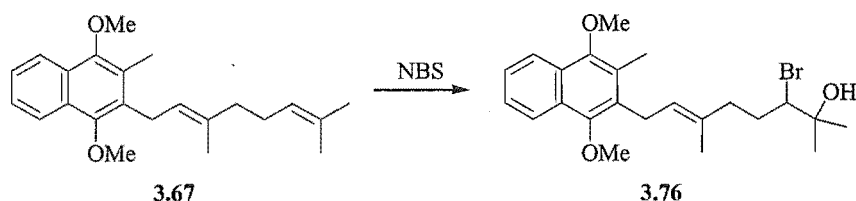
During work towards the total synthesis of tridentoquinone (**3.74**) in 1995, Brown and Robinson successfully functionalised a farnesyl intermediate (**3.75**) selectively at the terminal alkene (figure 3.7).<sup>40</sup> This synthesis involved bromohydrin formation followed by cyclisation to an epoxide and subsequent cleavage to the corresponding aldehyde. This method provides an alternative route to the desired regioselective formation of an epoxide at the terminal alkene of a polyisoprene chain, thereby overcoming problems encountered with earlier epoxidation procedures. It should be

noted that although this process increases the number of steps in the overall synthesis of the target analogues the efficiency of the synthesis is increased. The synthetic pathway devised for the preparation of target analogues **3.70** and **3.80** (see section 3.6) encompassed this approach.



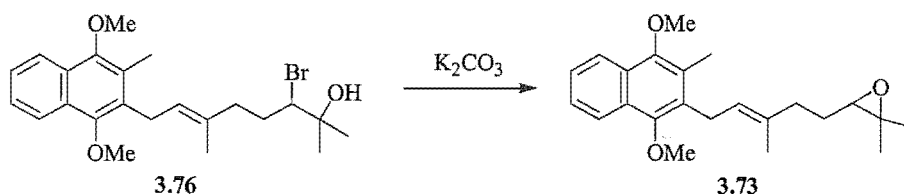
**Figure 3.7:** Structures of tridentoquinone (**3.74**) and a synthetic farnesyl intermediate (**3.75**)

The formation of the bromohydrin of **3.67** (scheme 3.23) was achieved by reaction with recrystallised *N*-bromosuccinimide, which afforded a ‘crude’ mixture of reaction products. Purification by radial chromatography afforded the desired bromohydrin **3.76** in 59% yield. The orientation of bromohydrin formation is determined by the substitution pattern of the alkene. The bromonium ion intermediate formed upon reaction of  $\text{Br}^+$  with the alkene is unsymmetrical, carrying more positive charge on the tertiary carbon centre than the secondary carbon centre. This promotes attack of the  $\text{OH}^-$  at the tertiary carbon centre giving the bromohydrin shown in scheme 3.23.



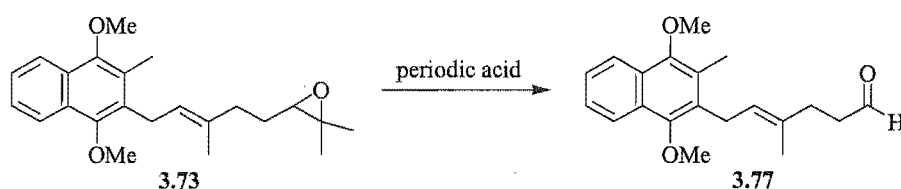
**Scheme 3.23**

Conversion of the bromohydrin (**3.76**) to the corresponding epoxide (**3.73**) (scheme 3.24) was achieved in 91% yield by reaction with potassium carbonate at room temperature for 18 hours. Upon work up this gave **3.73** which was pure by  $^1\text{H}$  NMR (c.f. scheme 3.22), and used without further purification.



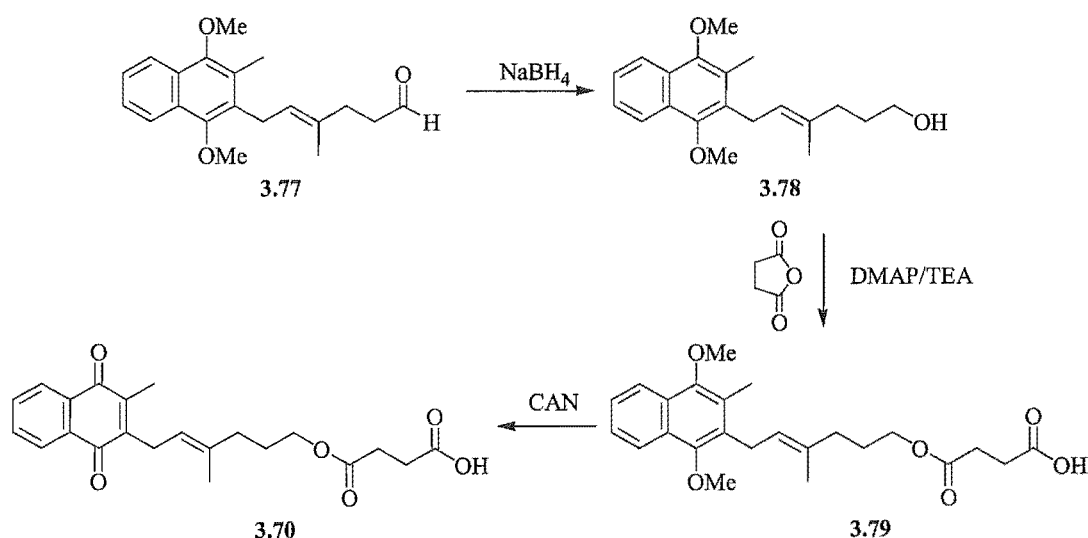
Scheme 3.24

Cleavage of the epoxide (3.73) with periodic acid in ether then afforded the corresponding aldehyde (3.77) in 60% yield without need for purification (scheme 3.25).



Scheme 3.25

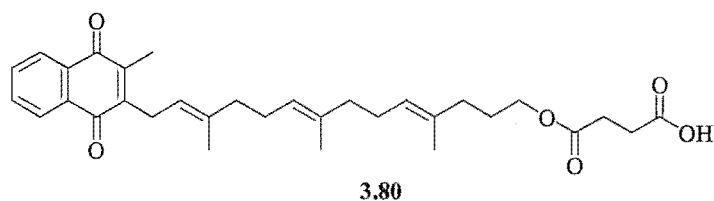
The synthesis of target analogue 3.70 from the aldehyde intermediate (3.77) was then achieved in three steps (scheme 3.26). The reduction of aldehyde 3.77 to the corresponding alcohol (3.78) proceeded in 60% yield, without need for purification. The addition of a linker to the alcohol to act as a spacer between the analogue and carrier protein is the next step in this synthesis. Although there are countless possible linkers available it was decided to use a succinyl based group as this was readily available and provided the required acid functionality for protein conjugation. The choice of base used for the coupling of succinic anhydride and the alcohol (3.78) proved important when considering both yield and ease of purification of this reaction. The use of pyridine as a base on reaction of 3.78 and succinic anhydride gave only 25% yield of the succinyl ester (3.79) and rigorous purification was required, but triethylamine has also been used in similar reactions and this proved more successful. Reaction of a solution of succinic anhydride, triethylamine and dimethylaminopyridine (DMAP) with the alcohol (3.78) for 18 hours, afforded the desired succinyl ester (3.79) in 60% yield with no purification required. Subsequent oxidative deprotection with CAN afforded the desired analogue 3.70 in 72% yield.

**Scheme 3.26**

The synthetic route outlined above has several advantages over literature procedures to similar compounds. Unlike the syntheses described by Watanabe *et al.*<sup>26-28</sup> (see section 3.3), this route does not require the coupling of a functionalised side-chain moiety which is produced via a multi-step synthesis. Instead the coupling reaction uses geranyl bromide, and functionality is introduced by manipulation of the terminal alkene of the coupled product (3.67). A similar approach was taken by Terao *et al.*<sup>30</sup> The synthetic route they established involved elongation of the side chain moiety by two carbon units during functionalisation. Although it is difficult to make a direct comparison between the two methods, as no data was given for yields in the literature, we believe that by removing the need for elongation of the side chain, and therefore reducing the number of synthetic steps, we have designed a more efficient synthesis of the target compounds. It may be noted that the same products can be arrived at via either route by using a starting material with one extra isoprene unit when proceeding via the synthetic method outlined above.

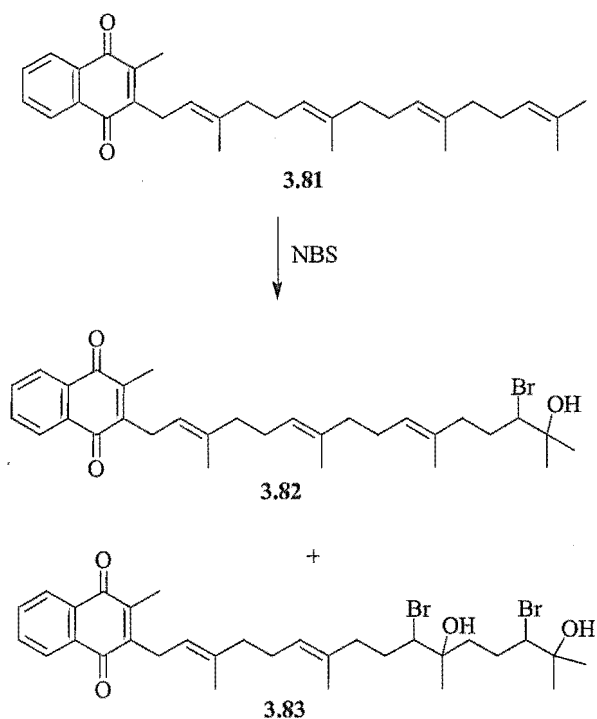
### 3.6 VITAMIN K<sub>2</sub> BASED ANALOGUES

Having established the synthetic route described above it was decided to use this in an attempt to produce analogues from vitamin K<sub>2</sub>. The target analogue (3.80) is shown in figure 3.8 and this was synthesised by an analogous route to that developed for the synthesis of 3.70.



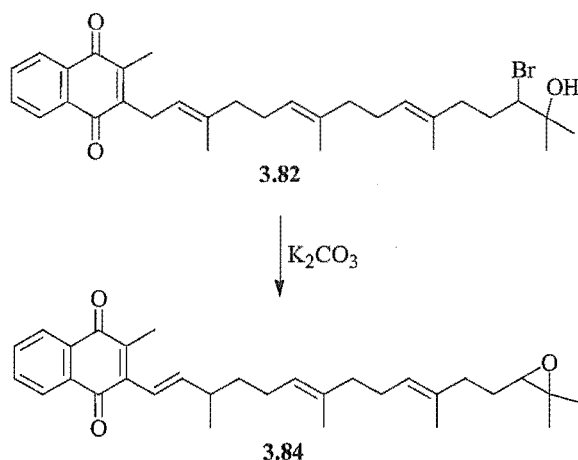
**Figure 3.8:** Structure of vitamin K<sub>2</sub> based analogue (3.80)

Initially this synthesis was attempted on vitamin K<sub>2</sub> itself, without protection of the quinone core. Reaction of vitamin K<sub>2</sub> (3.81) with *N*-bromosuccinimide afforded a mixture of products, which upon purification by radial chromatography afforded three fractions. By <sup>1</sup>H NMR analysis these were shown to contain recovered starting material (30 % yield), the desired bromohydrin (3.82) (47% yield) and the corresponding dibromohydrin product (3.83) (7% yield) (scheme 3.27). The signals of interest in the <sup>1</sup>H NMR spectrum of 3.82, are those corresponding to the terminal methyl groups and the proton adjacent to the bromine functionality. The presence of a doublet at 3.97 ppm was attributed to the proton adjacent to the bromine, on comparison with the corresponding proton (at 3.93 ppm) in 3.76 (scheme 3.23). The position of the terminal dimethyl group signals have shifted upfield from 1.57 and 1.58 ppm for vitamin K<sub>2</sub> to 1.32 and 1.33 ppm in 3.82 due to the presence of the adjacent hydroxyl group. The <sup>1</sup>H NMR spectrum of 3.83 was tentatively assigned by comparison, and showed a decrease in the integral of the allylic proton resonances at 4.91 and 5.17 ppm, and the two protons adjacent to the bromine groups are observed as a multiplet at 3.97 ppm. Regions of this spectrum were busy indicating the presence of a mixture of diastereoisomers.

**Scheme 3.27**

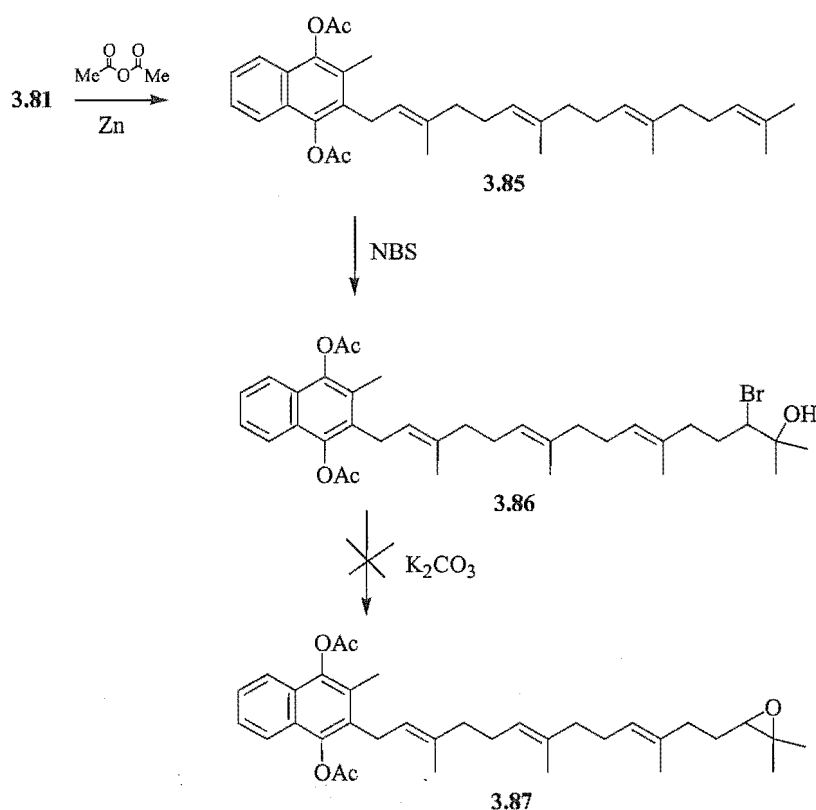
Oxidation of the bromohydrin (3.82) to the epoxide, with potassium carbonate, gave a crude product, which by  $^1\text{H}$  NMR analysis was inconsistent with the desired epoxide (scheme 3.28). The proton resonance of the benzylic protons at 3.35 ppm in the corresponding bromohydrin (3.82), was absent in the isolated product, and the number of allylic protons had increased from three to four by integration. From these findings it was postulated that the basic reaction conditions may have removed one of the acidic benzylic protons, leading to isomerisation of the  $\Delta^2$  double bond into conjugation with the aromatic ring (3.84). As this destroys the isoprenoid nature of the side chain it was decided not to proceed via this synthetic route and this was not purified further. This problem can be overcome by protection of the quinone nucleus.





### Scheme 3.28

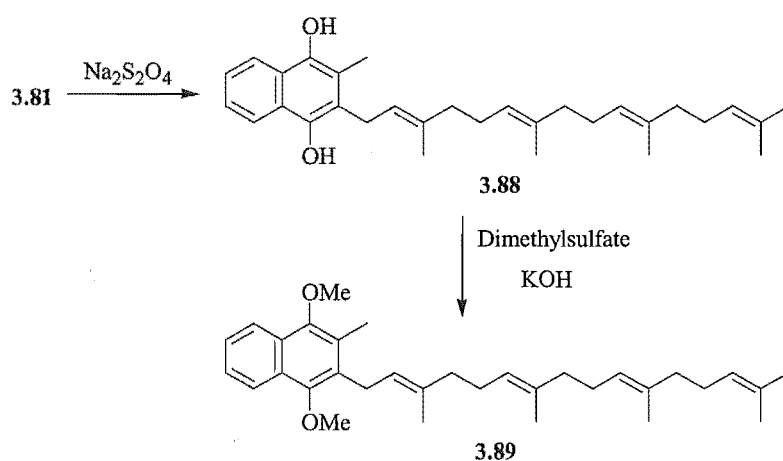
Refluxing vitamin  $\text{K}_2$  (**3.81**) in acetic anhydride in the presence of zinc powder followed by extraction into ethyl acetate afforded diacetoxyvitamin  $\text{K}_2$  (**3.85**) in 95% yield (scheme 3.29). Reaction of this with *N*-bromosuccinimide afforded the corresponding bromohydrin (**3.86**) in 15% yield. The reason for this low yield of bromohydrin is unknown, but similar results were not observed with any other substrate. Epoxidation of bromohydrin **3.86** with potassium carbonate again lead to a mixture of products. Upon comparison of the  $^1\text{H}$  NMR spectrum of the reaction mixture with the bromohydrin starting material (**3.86**), it was observed that the signal corresponding to the benzylic protons at 3.43 ppm in **3.86** was again absent, as observed in the case of unprotected vitamin  $\text{K}_2$ . In addition the two singlets at 2.46 and 2.48 ppm in the bromohydrin (**3.86**), corresponding to the acetyl protecting groups, were also absent in the isolated product. From this evidence it was postulated that the acetyl protecting groups had been cleaved in the basic reaction conditions and the  $\Delta^2$  double bond had again moved into conjugation with the aromatic ring. As isomerisation of the double bond lead to a compound which could not be utilised, this synthetic route was not further investigated.



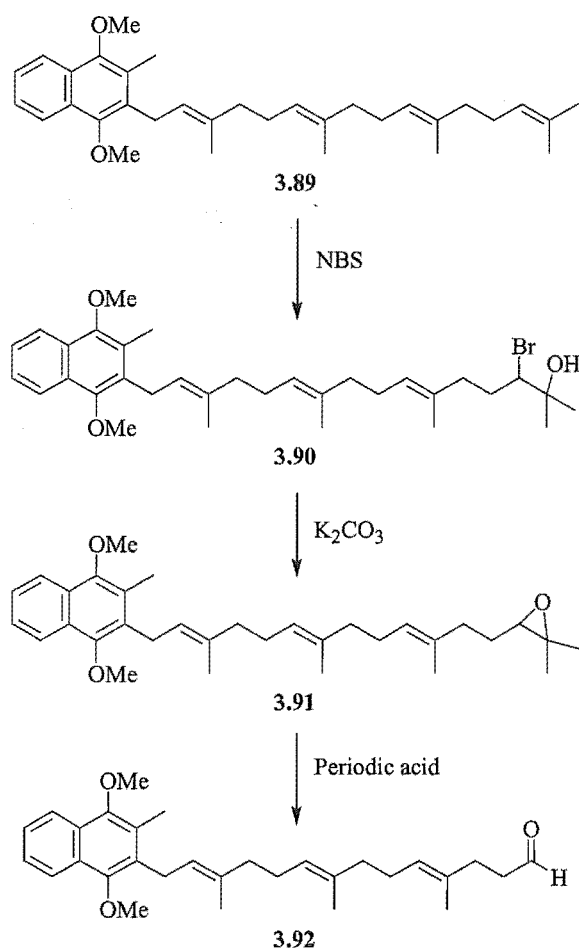
### Scheme 3.29

As the methoxy ether protecting group had been shown in earlier work to withstand the reaction conditions of the epoxidation, and subsequent steps of this synthetic approach, it was decided to use this protecting group for vitamin K<sub>2</sub>. Initially the same method as used in the preparation of the naphthoquinone core compound 3.11 (see section 3.4.1) was attempted. The reduction of vitamin K<sub>2</sub> with stannous chloride and concentrated hydrochloric acid afforded only starting material upon work up. The reduction of vitamin K<sub>2</sub> (3.81) with sodium dithionite under a nitrogen atmosphere in degassed solvents was then attempted. This also led to the isolation of vitamin K<sub>2</sub> upon work-up and <sup>1</sup>H NMR analysis. This reaction was repeated and followed by <sup>1</sup>H NMR analysis in order to establish if any dihydrovitamin K<sub>2</sub> (3.88) could be detected (scheme 3.30). After a reaction time of 30 minutes, an aliquot was removed from the reaction vessel, dried under reduced pressure and analysed by <sup>1</sup>H NMR. At this point the ratio of dihydrovitamin K<sub>2</sub> to vitamin K<sub>2</sub> was 75:25, by integration of the benzylic proton resonances which appear as two doublets at 3.36 ppm for the quinone and 3.53 ppm for the hydroquinone. Analysis of aliquots in this same manner after reaction time of 60, 90, 150 and 210 minutes gave variable results, with the hydroquinone:quinone ratio ranging from 60:40 at 90 minutes, back

to 75:25 at 150 minutes. From these results it appeared that exposure to air may be responsible for the observed variation in hydroquinone:quinone ratio. At 210 minutes two aliquots were taken, with one being exposed to air for longer before  $^1\text{H}$  NMR analysis. The hydroquinone:quinone ratios in this case were 70:30 for the control experiment, and only 45:55 for the sample exposed to air. This supported the earlier hypothesis of reoxidation of dihydrovitamin  $\text{K}_2$  to vitamin  $\text{K}_2$  upon exposure to the atmosphere. From this finding all further attempts to synthesise dimethoxyvitamin  $\text{K}_2$  (**3.89**) were carried out without isolation of the hydroquinone intermediate. If the hydroquinone was immediately transferred via cannular to dimethylsulfate and reacted with potassium hydroxide, a mixture of dimethoxyvitamin  $\text{K}_2$  (**3.89**) and vitamin  $\text{K}_2$  (**3.81**) was isolated. These could be separated by radial chromatography to yield dimethoxy-vitamin  $\text{K}_2$  in 33% yield, with 15% recovery of vitamin  $\text{K}_2$ . It has been shown by Kraus and On Man<sup>41</sup> that the use of phase transfer catalysts can increase the yield of the reductive methylation of quinones. This process removes the need for the transfer of the highly air sensitive dihydrovitamin  $\text{K}_2$  by having the reduction and methylation occurring in one flask. A solution of sodium dithionite was added to a solution containing vitamin  $\text{K}_2$  and tetrabutylammoniumiodide. The addition of potassium hydroxide followed by dimethylsulfate and reaction for 18 hours, afforded after purification, the desired dimethoxyvitamin  $\text{K}_2$  (**3.89**) in 63% yield (scheme 3.30).

**Scheme 3.30**

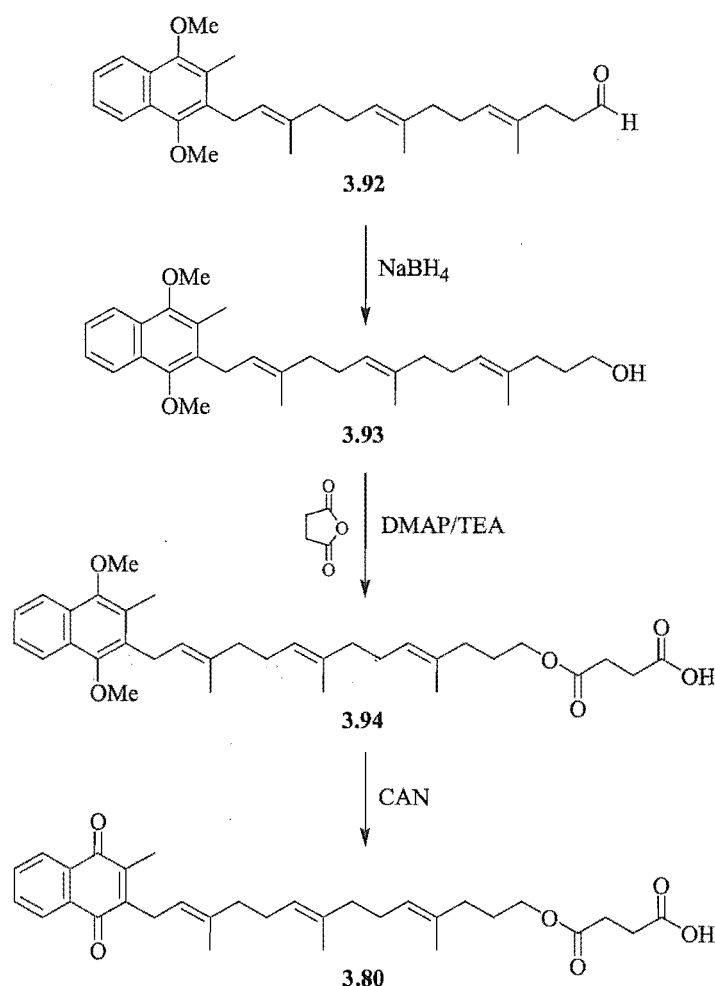
Having successfully prepared dimethoxyvitamin K<sub>2</sub> (**3.89**) the target analogue **3.80** could now be synthesised by the same route used previously (scheme 3.31). Reaction of dimethoxyvitamin K<sub>2</sub> (**3.89**) with *N*-bromosuccinimide afforded the bromohydrin **3.90** in 50% yield after purification by radial chromatography. Epoxidation with potassium carbonate yielded the corresponding epoxide (**3.91**) in 80% yield with no purification required. The presence of a doublet corresponding to the benzylic proton resonance, at 3.57 ppm in the <sup>1</sup>H NMR spectrum of **3.91** implied that no isomerisation of the Δ<sup>2</sup> double bond had occurred in this case. Cleavage of the epoxide (**3.91**) with periodic acid yielded the corresponding aldehyde (**3.92**) in 95% yield.



**Scheme 3.31**

Reduction of the aldehyde of vitamin K<sub>2</sub> (**3.92**) with sodium borohydride to give the alcohol (**3.93**) (scheme 3.32) proceeded cleanly in 74% yield. The addition of the succinyl linker with succinic anhydride, dimethylaminopyridine and triethylamine

afforded **3.94** in 60% yield with no purification. Deprotection of the quinone core with CAN gave the target analogue **3.80** in 60% yield.



**Scheme 3.32**

In summary, attempts to synthesise **3.80** from vitamin K<sub>2</sub> or diacetoxyvitamin K<sub>2</sub> were unsuccessful due to isomerisation of the  $\Delta^2$  double bond being observed in the basic reaction conditions. However, the preparation of dimethoxyvitamin K<sub>2</sub> (**3.89**) via reductive methylation using a phase transfer catalyst overcame this problem, leading to the successful completion of this synthesis via the route established for the preparation of **3.70**.

This study resulted in the synthesis of two novel side chain functionalised analogues of vitamin K (**3.70** and **3.80**), suitable for conjugation to a protein for immunological studies. We found that side chain functionalisation of a polyisoprene naphthalene was the most efficient route to these compounds, as attempts to couple a functionalised side chain to the naphthalene core lead to isolation difficulties of the final product.

Both of the vitamin K analogues synthesised (**3.70** and **3.80**) were then used at Canterbury Health Laboratories for initial studies into the production of monoclonal antibodies, for use in the development of an immunoassay for vitamin K in blood and breast milk.

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## **CHAPTER FOUR**

### **IMMUNOLOGICAL STUDIES**

## 4.1 IMMUNOASSAYS

Immunoassays are recognised as a quick and efficient method for screening large numbers of biological samples. Due to the decreased preparation time of samples and high-throughput capability an immunoassay for vitamin K levels in blood is an attractive target. There are many different types of immunoassays, including radioimmunoassays and enzyme-linked immunosorbent assays (ELISAs). Both of these assay types utilise the same basic principles, while differing in the detecting methods for quantitation. In both cases an antibody is produced which recognises the antigen of interest, in this case vitamin K. This antibody is added to a blood sample where it binds the relevant antigen. A second antibody is then introduced which will bind to the first antibody. If this second antibody is radiolabelled, the assay is termed a radioimmunoassay, and the amount of radiolabel bound by the sample gives a measure of the amount of antigen in the original sample. In the case of an ELISA, the second antibody is labelled with an enzyme. Introduction of the enzyme substrate produces a coloured product which, when detected spectrophotometrically gives a measure of the antigen concentration in the sample. For the purposes of our study the ELISA was the target assay.

The first antibody introduced in the ELISA, which binds to the antigen (vitamin K), is a monoclonal antibody. This is an antibody that is designed and produced to recognise a specific antigen. Therefore, in order to develop a functional ELISA it is first necessary to produce monoclonal antibodies which will bind vitamin K.

### 4.1.1 MONOCLONAL ANTIBODY PRODUCTION

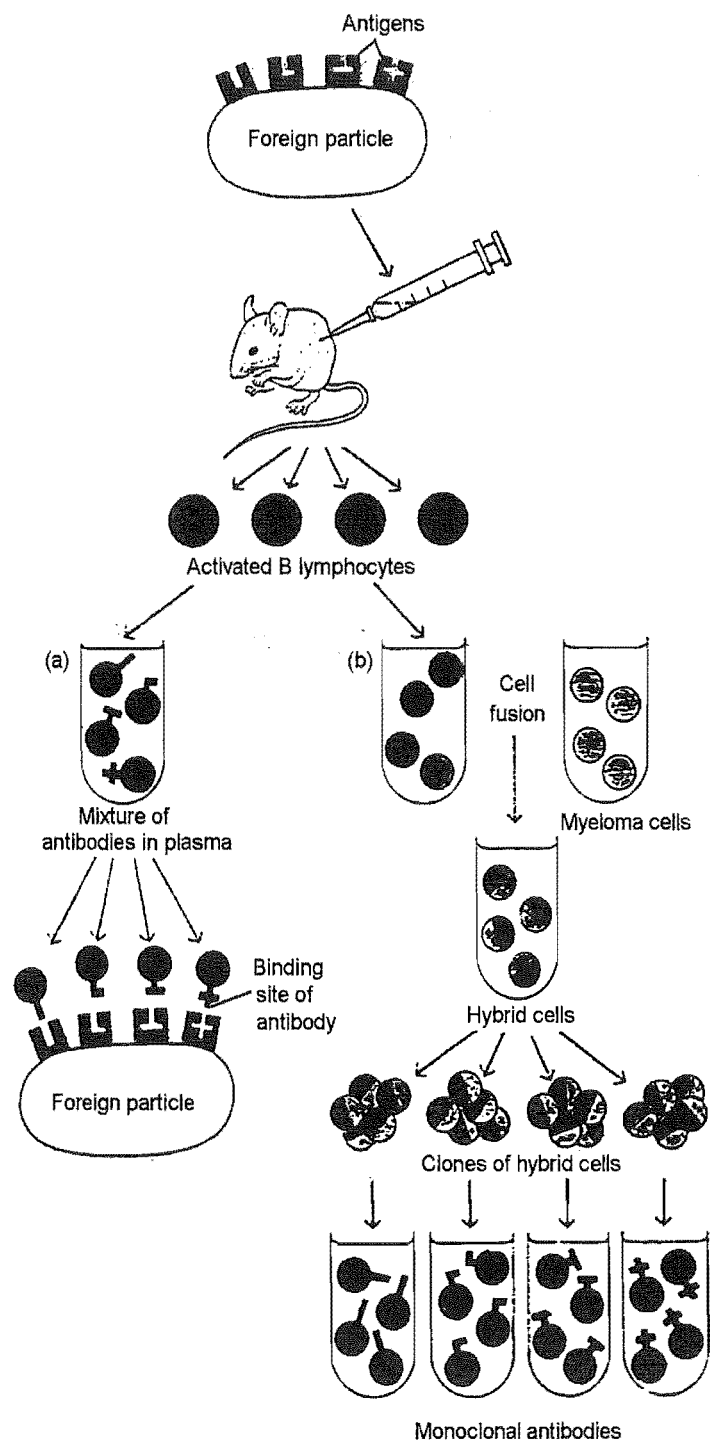
Monoclonal antibodies do not differ structurally from naturally occurring antibodies.<sup>1</sup> What makes monoclonal antibodies (MCAs) unique is that all antibodies produced in any single preparation are identical.<sup>1</sup> In general, when the body is exposed to a foreign molecule (or antigen) an immune response is triggered.<sup>1</sup> Our study requires an antibody to recognise vitamin K as the antigen. However, vitamin K itself is unable to evoke an immune response for several reasons. Most importantly the K vitamins are naturally occurring compounds, and therefore will not be recognised as a foreign particle. Vitamin

K is also a small molecule which is not readily soluble in aqueous environments, again affecting its immunogenicity. The conjugation of vitamin K to a carrier protein will increase both the particle size and aqueous solubility. The carrier protein is chosen to be recognised as a foreign body by the host, thereby evoking an immune response. This immune response involves the production of specific proteins called antibodies, which link to the antigen by a series of non-covalent bonds.<sup>1</sup> Antibodies are produced by B-lymphocytes, with each B-lymphocyte producing only one specific type of antibody, as stated in the 'one-cell, one-antibody' axiom of Burnet's clonal selection theory.<sup>2</sup> Any single antigen will usually elicit a polyclonal response with different antibodies directed to different antigenic determinants (or epitopes), or even different combining sites of the same epitope\*.<sup>2</sup> In the case of the vitamin K-carrier protein 'antigen', this implies antibodies will be produced to various surface features of the carrier protein, and hopefully to the vitamin K core attached to the protein surface.

In the production of MCAs the aim is to produce an immortal cell line producing an antibody which recognises the epitope of choice for a given antigen, i.e. the vitamin K naphthoquinone core. The overall process for MCA production is outlined in figure 4.1. A mouse is inoculated with the vitamin K-carrier protein conjugate and after some time the B-lymphocytes are harvested by isolation from the spleen. As B-lymphocytes cannot be grown in culture conditions, they are then fused with myeloma cells which are lacking either hypoxanthine phosphoribosyl transferase (HPRT) or thymidine kinase.<sup>2</sup> Myeloma cells are a strain of rapidly dividing cells that may be readily grown in culture conditions. Both HPRT and thymidine kinase are essential for the salvage pathway of DNA synthesis, and this is important in the selection of fused hybrid cells. The cell culture, after fusion, will contain fused and unfused myeloma cells, as all unfused B-lymphocytes will not survive culture conditions.

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\* An epitope is a site on a large molecule against which an antibody will be produced and to which it will bind.



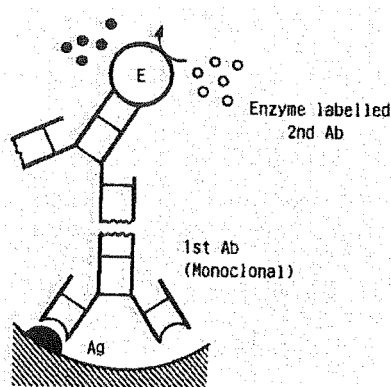
**Figure 4.1:** Schematic representation of the process for MCA production

This cell culture is grown in a medium containing hypoxanthine, aminopterin and thymidine (HAT).<sup>1</sup> Aminopterin blocks the main pathway for purine and pyrimidine biosynthesis and cells which possess HPRT can utilise hypoxanthine to synthesise purines and thymidine to synthesise pyrimidines via a salvage pathway. As the myeloma cells are HPRT negative they will die under these culture conditions, and only cells which have successfully undergone fusion, and therefore are HPRT positive, will survive.<sup>1</sup> This fusion will result in a cell which can grow in culture and produce antibodies to an epitope of the vitamin K-carrier protein conjugate.

The successfully fused cells are then diluted, separated to single cells, and grown in culture to give a series of cell cultures each producing a single antibody (MCA).<sup>1</sup> Each culture is screened for antibodies which recognise vitamin K, and the required cell line can be cultured for further antibody production. These antibodies may then be used in an ELISA for the determination of vitamin K levels in blood.

#### 4.1.2 ENZYME-LINKED IMMUNOSORBENT ASSAY

The principles of an ELISA are quite simple (see figure 4.2). The general procedure for an ELISA initially involves a sample containing the antigen, vitamin K, being placed in a microtitre plate. The MCA is introduced to the well and any unbound MCA is washed out.<sup>3</sup> A second MCA is introduced which will bind to the first antibody, and is coupled to an enzyme.<sup>3</sup> The introduction of the substrate of this enzyme will lead to the production of a coloured product which may be detected spectrophotometrically.<sup>3</sup> Although the ELISA is less sensitive than some alternative techniques, including radioimmunoassay, it boasts simpler sample handling and faster measurement, especially in automated systems.<sup>2</sup> Once developed an ELISA could potentially be used to spectrophotometrically determine the vitamin K content of serum samples, reading a 96 well microtitre plate in minutes, as opposed to current HPLC methods which require extensive sample preparation and have an analysis time of approximately 30 minutes per sample.



**Figure 4.2:** Schematic of an ELISA system<sup>3</sup>

## 4.2 INITIAL IMMUNOLOGY FINDINGS

Following the successful synthesis of analogues **3.70** and **3.80** (see chapter 3), these were taken to Dr Peter Elder of the Steroid Laboratory at Canterbury Health Laboratories for immunology studies. These compounds were coupled to bovine serum albumin (BSA) and bovine thyroglobulin (Tg). BSA was used as the carrier protein for inoculation and the Tg conjugate was used in the antibody screening process. The coupling procedure used in this instance was a mixed anhydride method. A sample of 3 mg of the analogue was treated with 10  $\mu$ L of tributylamine and 4  $\mu$ L of isobutyl chloroformate, before addition to 10 mg of BSA or Tg. Upon completion of this reaction it was noted that some yellow oil remained unreacted and adhered to the reaction vessel. This observation led to model peptide coupling studies to investigate the effectiveness of the conjugation of a vitamin K analogue (**3.70** or **3.80**) and a protein (see section 4.3). The crude analogue-BSA conjugates were then used for inoculation of mice, as purity of the antigen is not important for antibody production.<sup>2</sup> An initial tail bleed of these mice at 6 weeks showed that antibodies were being produced and after 8 weeks the spleen cells were harvested. The antibodies produced after inoculation with the analogue-BSA conjugate will recognise sites on both BSA and the vitamin K analogue. The antibodies harvested were screened for activity towards vitamin K. To achieve this a sample of mouse blood was placed in a microtitre plate containing the analogue-Tg conjugate. Any antibodies recognising the analogue, which are present in the blood, will bind to the

analogue-Tg conjugate and may be visualised by the addition of antimouse HRP antibodies. These antimouse HRP antibodies will bind to the antibodies in mouse blood, and upon addition of 3,3',5,5'-tetramethylbenzidine produce a blue colour. Testing via this method eliminates the possibility of detecting antibodies specific to the carrier protein BSA, as there is no BSA present in the test medium. Although tests showed that some analogue specific antibodies had been produced, all efforts to bind these antibodies with vitamin K (e.g. menadione) proved unsuccessful. From these results it was postulated that the 'bridge effect' must have been occurring. This involves antibodies recognising both the vitamin K analogue and the chemical bridge used for conjugation to BSA, in this case the isoprene side chain and linker moiety, as opposed to the naphthoquinone core. With this in mind further analogues of vitamin K were designed which did not possess the long isoprenoid side chain (see section 4.4).

### 4.3 MODEL COUPLING STUDIES

Following observations by Dr Peter Elder during the conjugation studies, that some organic residue remained after coupling, model studies to investigate this coupling process were undertaken. The vitamin K analogues synthesised (3.70 and 3.80) possess a carboxylic acid functionality to allow coupling to a protein via peptide bond formation with the amine group of the lysine side chain. The model studies must, therefore, be carried out using lysine or short lysine containing peptides. For this reason the commercially available *N*- $\alpha$ -acetyl-lysine and *N*-acetyl-glycine-lysine methyl ester were used. The use of amino acids protected at the  $\alpha$  amine was essential to block reaction of the vitamin K analogue at this site.

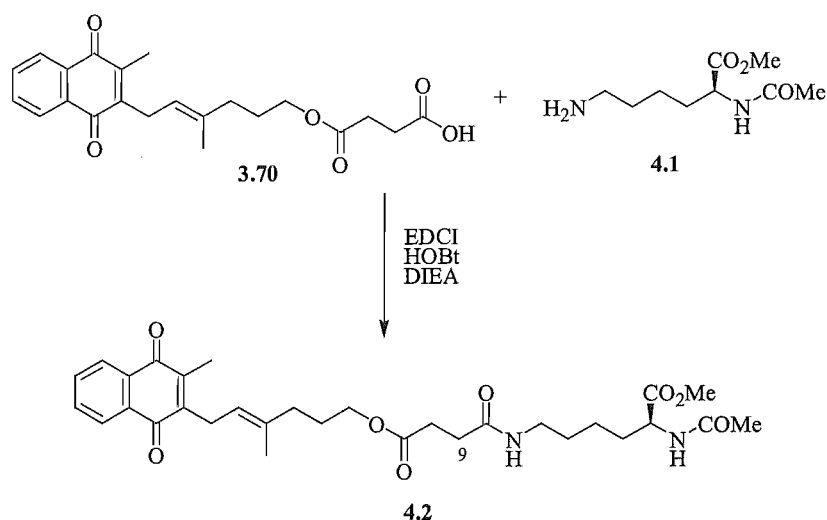
Initially the peptide coupling method chosen for these studies was the mixed anhydride method, as this had been used for protein conjugation. However, reaction of 3.70 and *N*- $\alpha$ -acetyl-lysine methyl ester via the mixed anhydride method was hampered by solubility problems. The preparation of a solution of *N*- $\alpha$ -acetyl-lysine methyl ester and triethylamine in DMF proved difficult, with some solid remaining after gentle heating and sonication. This would suggest that this method is not ideal for the protein conjugation studies. After addition of this to a solution containing the analogue (3.70),

*N*-methyl morpholine, and isobutyl chloroformate at  $-15\text{ }^{\circ}\text{C}$ , the reaction was allowed to warm slowly to room temperature. Subsequent work-up and analysis of the crude product by  $^1\text{H}$  NMR analysis showed no indication of a successful coupling. The  $^1\text{H}$  NMR spectrum showed only the resonances of **3.70**, with no resonances corresponding to the amino acid moiety of the coupled product. As the mixed anhydride coupling had proved unsuccessful, EDCI was used in all further studies.

### 4.3.1 COUPLING TO PROTECTED LYSINE

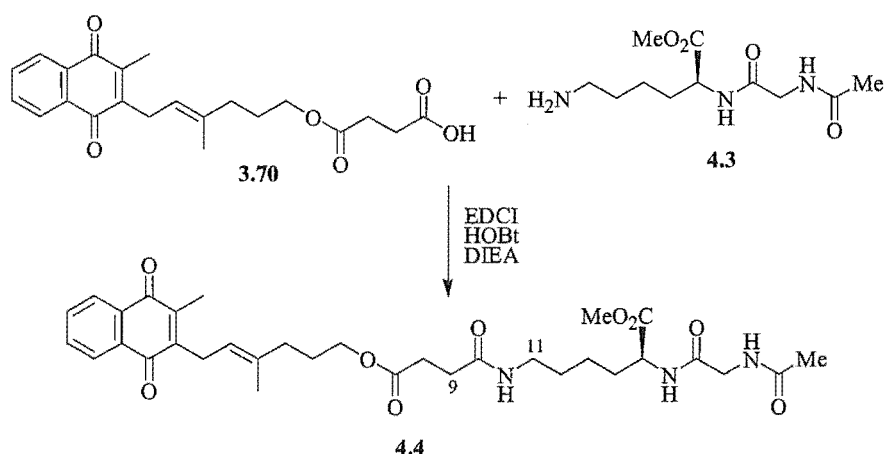
The peptide coupling method used for these studies utilised the coupling reagent 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) see scheme 4.1.<sup>4</sup> The first EDCI coupling was attempted using an *N*- $\alpha$ -protected lysine residue (**4.1**), as it is the side chain amino group of lysine, and not the  $\alpha$  amine, that is the target point for conjugation to the carrier protein. The coupling reaction consisted of the addition of EDCI, 1-hydroxybenzotriazole hydrate, and diisopropylethylamine to a solution of the analogue (**3.70**) and *N*- $\alpha$ -acetyl-lysine methyl ester (**4.1**) in dichloromethane. After reaction for 18 hours the crude product was isolated and purified by radial chromatography. The desired product (**4.2**) was isolated in 55% yield and analysed by  $^1\text{H}$ ,  $^{13}\text{C}$  and two-dimensional NMR spectroscopy.



**Scheme 4.1**

Analysis of **4.2** by  $^1\text{H}$  and  $^{13}\text{C}$  NMR indicated that conjugation of **3.70** and lysine had been successful. The upfield shift of the C-9 methylene proton resonance from 2.63 ppm in **3.70**, to 2.43 ppm in **4.2** is consistent with a change in functionality from an acid to an amide adjacent to this position. The presence of  $^1\text{H}$  resonances for the lysine residue was also apparent. The  $^1\text{H}$  NMR spectrum of **4.2** (see figure 4.3), showed a series of multiplets corresponding to the methylene proton resonances of the lysine side chain at 1.35, 1.51, 1.71, 1.82, 3.20 and 3.26 ppm. The  $\alpha$  proton resonance of lysine was observed as a multiplet at 4.56 ppm, while the methyl ester protons appeared as a singlet at 3.74 ppm. The acetyl-protecting group of the lysine appeared as a singlet at 2.04 ppm, and finally the two NH protons were present at 5.94 and 6.37 ppm. Observation of a molecular ion ( $\text{MH}^+$ ) at 569.2871 was also consistent with the desired product **4.2**.



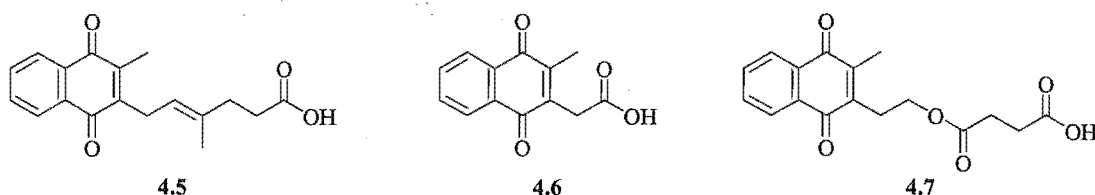
**Scheme 4.2**

Analysis of the isolated product by  $^1\text{H}$  NMR spectroscopy showed several signals consistent with the desired conjugate **4.4**. The methylene resonances of the succinyl ester shifted from 2.58 and 2.63 ppm in the **3.70**, to 2.44 and 2.62 ppm in the conjugated product. The shift in C-9 proton resonance is consistent with its change in environment due to the adjacent functionality being converted from an ester to an amide. The three resonances at 6.25, 6.74 and 7.04 ppm were consistent with the presence of three amide protons in **4.4**. The methylene resonance of glycine is observed as a multiplet at 3.95 ppm, while the C-11 proton resonances of the lysine side chain appear as a pair of multiplets at 3.18 and 3.26 ppm. The remaining proton resonances of the lysine side chain are observed upfield as multiplets at 1.32 (2H), 1.49 (2H), 1.70 (1H) and 1.85 (1H) ppm. Finally the lysine  $\alpha$  proton resonance is a multiplet at 4.53 ppm.

Modelling of the coupling reaction of a vitamin K analogue (**3.70**) with simple amino acids or dipeptides has shown that the mixed anhydride peptide coupling method is not suited to this reaction. Model couplings of **3.70** and *N*-acetyl-lysine methyl ester via the mixed anhydride method showed no coupled product, whereas the same reaction using EDCI coupling conditions proceeded to give the coupled product **4.2** in 55% yield.

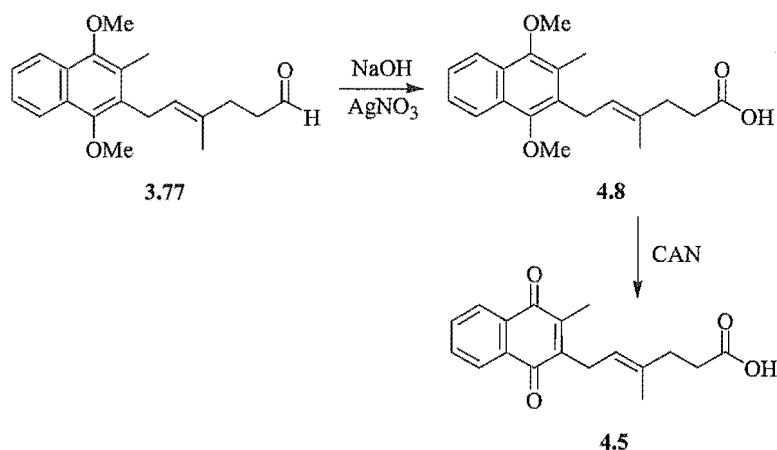
## 4.4 DESIGN AND SYNTHESIS OF FURTHER VITAMIN K ANALOGUES

The results of initial immunology studies (see section 4.2) suggested that attempts to raise antibodies to recognise the quinone moiety of vitamin K may have been hampered by the 'bridge effect'. This means that antibodies produced by the host recognise a linker between the desired antigenic moiety (in this case the quinone core of vitamin K analogues **3.70** or **3.80**) and the carrier protein (BSA). In an attempt to overcome this problem three further vitamin K analogues were designed which did not possess the natural long isoprene side chain of vitamin K. The three analogues designed (see figure 4.4) did, however, retain the carboxylic acid functionality to allow conjugation to a carrier protein for immunological studies, and the 2-methyl-1,4-naphthoquinone core.

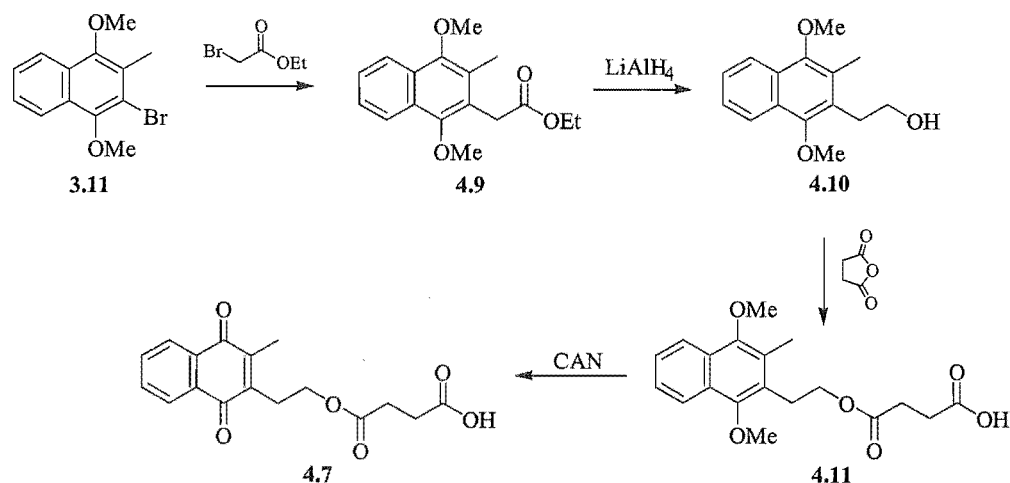


**Figure 4.4** Structures of short chain target analogues

Analogue **4.5** has previously been synthesised by Masaki *et al.*<sup>5</sup> via hydrolysis of the corresponding methyl ester, which was prepared from an allylic sulfide by sulfur-contractive anionic [2,3]-sigmatropic rearrangement. This synthesis had proceeded in 15% yield, and afforded a mixture of the *cis* and *trans* isomers. It was recognised, however, that this analogue could be synthesised in two steps (scheme 4.3) from **3.77**, an intermediate in the synthesis of **3.70** discussed in chapter 3. The aldehyde (**3.77**) was directly oxidised to the corresponding carboxylic acid (**4.8**) in 50% yield, using silver nitrate and sodium hydroxide. This carboxylic acid was then deprotected with CAN to afford **4.5** in 60% yield after purification by radial chromatography.

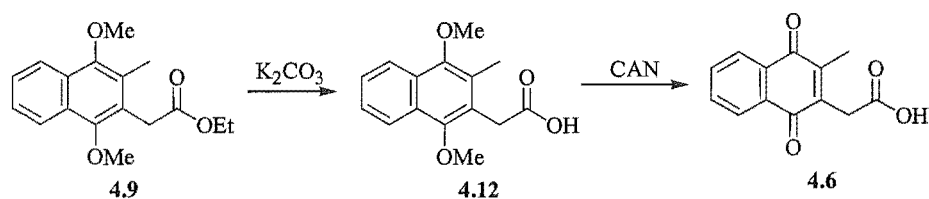
**Scheme 4.3**

The synthesis of analogues 4.6 and 4.7 began with the same brominated core compound (3.11) used in the earlier synthesis of analogues 3.70. This protected quinone (3.11) was coupled to ethylbromoacetate using a cuprate coupling (see section 3.4.3) to give 4.9 (scheme 4.4). The yield from this coupling reaction, after purification by radial chromatography, was found to be 35%, which is much lower than the 80% yield observed using analogous reaction conditions with geranyl bromide. A similar lowering in yield for the cuprate coupling reaction was observed by Chenard *et al* when comparing the coupling of methylbromoacetate to a quinone core, with the analogous reaction using allylic bromides.<sup>6</sup> Reduction of 4.9 with lithium aluminium hydride afforded the corresponding alcohol (4.10) in 90% yield as a white solid after radial chromatography. This was then reacted with succinic anhydride in the presence of *N,N*-dimethylaminopyridine and triethylamine to give the succinyl ester (4.11) in 55% yield. Oxidative deprotection of the quinone with CAN and radial chromatography, afforded analogue 4.7 in 75% yield.



Scheme 4.4

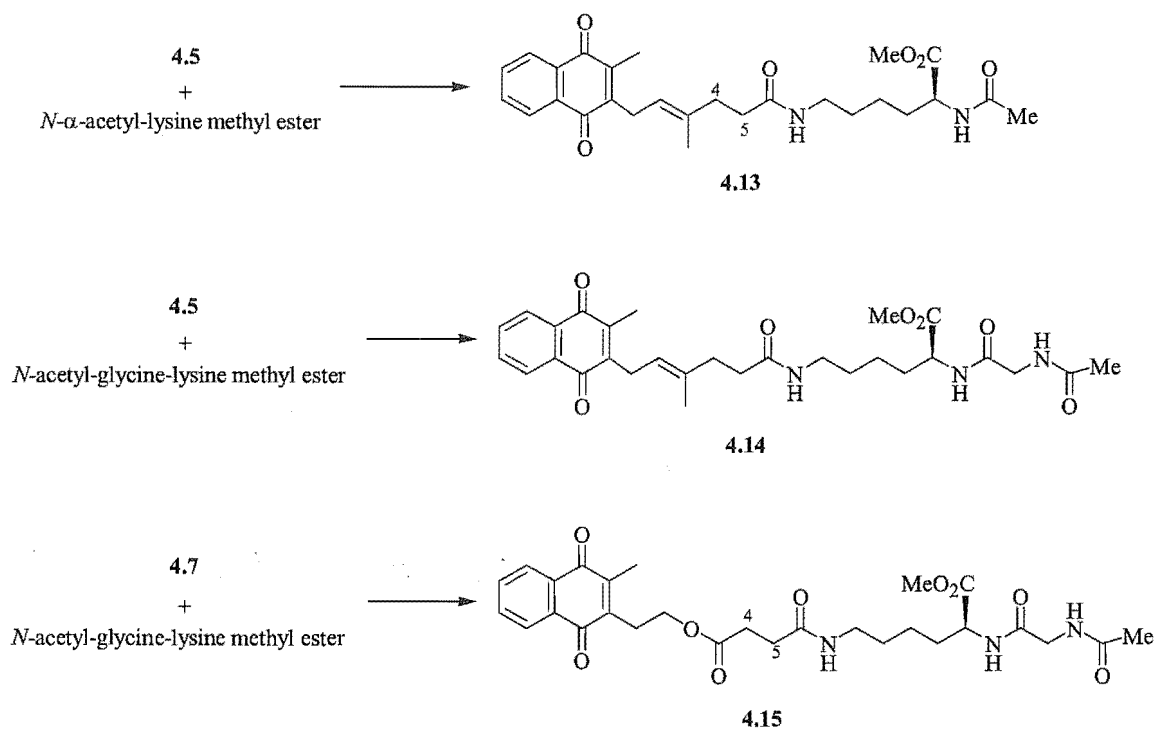
The synthesis of analogue 4.6 proceeded in two steps (scheme 4.5) from the ester 4.9 used in the synthesis of analogue 4.7. Hydrolysis of the ethyl ester with potassium carbonate in refluxing methanol afforded the corresponding carboxylic acid (4.12) in 73% yield. The oxidative deprotection of 4.12 using  $\text{CAN}$ , although attempted several times, always gave a complex mixture of products. All attempts to purify this mixture by radial chromatography or recrystallisation proved unsuccessful. Due to time constraints further investigation of this step were not possible.



Scheme 4.5

Following the successful synthesis of analogues 4.5 and 4.7, these were used in model coupling reactions with *N*-acetyl-lysine and *N*-acetyl-glycine-lysine methyl ester. These reactions were used to establish that peptide coupling between the carboxylic acid functionality of analogues 4.5 and 4.7, and the amine group of a lysine residue was possible. Analogue 4.5 was coupled to *N*- $\alpha$ -acetyl-lysine methyl ester, while both 4.5 and 4.7 were coupled to the dipeptide *N*-acetyl-glycine-lysine methyl ester (scheme 4.6) using the EDCI coupling method outlined earlier (see section 4.3.1). Following extraction and purification by radial chromatography coupled products 4.13, 4.14, and

**4.15** were isolated in 51%, 31% and 85% yield respectively. These products were analysed by NMR spectroscopy and mass spectrometry.



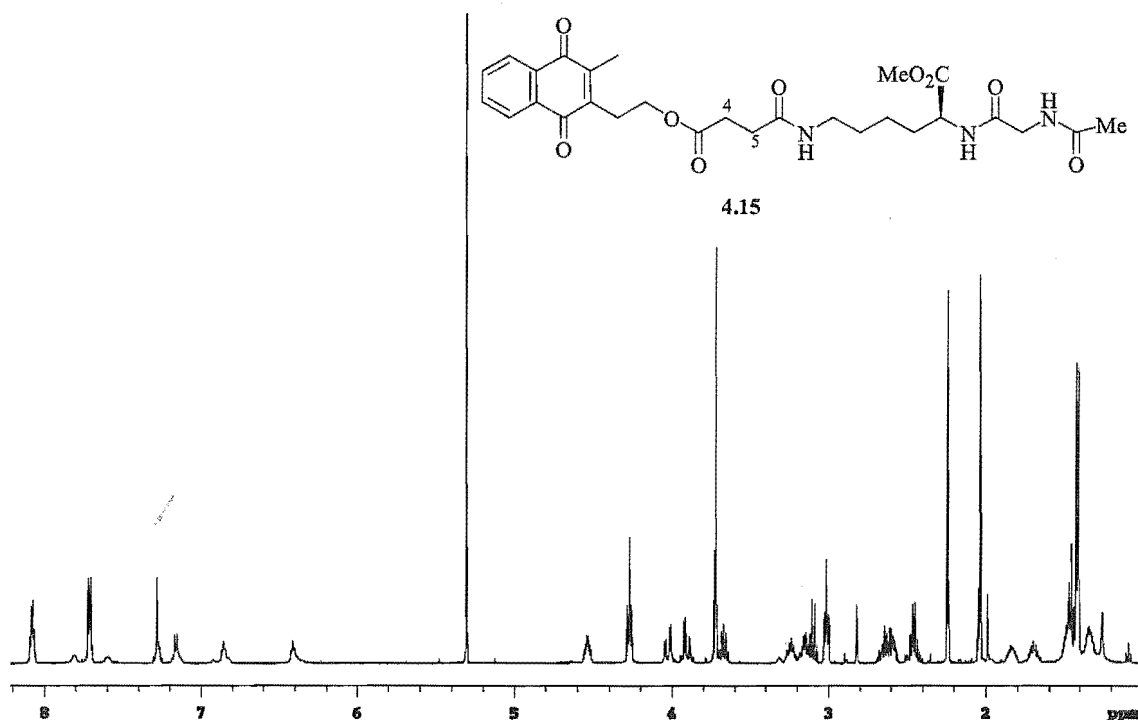
**Scheme 4.6**

Analysis of **4.13** by  $^1\text{H}$  NMR spectroscopy showed several resonances indicative of successful coupling. The resonances for protons at C-4 and C-5, which are observed at 2.29 and 2.41 ppm respectively in **4.5**, overlap in the conjugated compound to give a multiplet at 2.29 ppm. This is due to the change in chemical environment of the protons at C-5 following the conversion of the adjacent acid functionality in **4.5**, to an amide in **4.13**. The presence of two amide resonances at 5.72 and 6.32 ppm, and the resonance of the  $\alpha$ -proton of lysine at 4.54 ppm are also indicative of the presence of the desired product **4.13**. The terminal methylene resonance of the lysine side chain was observed as a multiplet at 3.17 ppm, while the other lysine side chain resonances were observed at 1.28, 1.46, 1.68 and 1.80 ppm. A molecular ion ( $\text{M}+\text{H}$ ) observed at 483.25 by mass spectrometry, is also consistent with **4.13**.

Similar results were observed in the  $^1\text{H}$  NMR analysis of conjugate **4.14**. The resonances for the protons at C-4 and C-5 at 2.29 and 2.41 ppm respectively in **4.5**, are seen as a single multiplet at 2.29 ppm in **4.14**. The terminal methylene resonance of the

lysine side chain is observed as a broad multiplet at 3.12 ppm, which is consistent with results from the analogue-lysine conjugate (**4.13**). Differences observed in this spectrum, when compared with that of **4.13**, include a multiplet at 3.92 ppm for the methylene of glycine, and the presence of three amide proton resonances at 6.17, 6.80 and 7.10 ppm.

The coupling of analogue **4.7** to the protected glycine-lysine dipeptide also proceeded smoothly. The  $^1\text{H}$  NMR of the conjugated product (see figure 4.5) showed a downfield shift of proton resonances of C-4 and C-5 to 2.61 ppm from 2.55 ppm in **4.7**. The methylene adjacent to the side chain amine of lysine is observed as two distinct multiplets at 3.14 and 3.24 ppm. The methylene protons of glycine are observed as a multiplet at 3.95 ppm, and the  $\alpha$ -proton resonance of lysine is seen as a multiplet at 4.54 ppm. Finally the three amide proton resonances are observed at 6.41, 6.86, and 7.16 ppm. The observation of a molecular ion ( $\text{M}+\text{H}$ ) at 558.25 is consistent with the coupled product **4.15** ( $\text{C}_{28}\text{H}_{36}\text{N}_3\text{O}_9$ ).



**Figure 4.5:**  $^1\text{H}$  NMR spectrum of conjugate **4.15**

Two further analogues of vitamin K have been successfully synthesised for use in further immunological studies. A new synthetic route to **4.5** has been developed which is higher yielding than the reported synthesis,<sup>5</sup> and produces purely the desired *trans*



isomer. Analogue 4.7 is a new compound prepared via a simple three step synthesis from a protected quinone core 3.11 and ethylbromoacetate. These two analogues have successfully been coupled to *N*-acetyl-lysine and *N*-acetyl-glycine-lysine methyl ester in model studies for their conjugation to carrier proteins during immunology studies.

## 4.5 FURTHER IMMUNOLOGY STUDIES

The second series of vitamin K analogues (4.5 and 4.7) were also forwarded to Dr Peter Elder for immunology studies. The coupling of 4.5 and 4.7 to BSA and Tg via a mixed anhydride coupling encountered similar solubility problems to those observed during the coupling of 3.70 and 3.80. However, the crude products from the conjugation of these analogues with BSA were used for the inoculation of mice. After 6 weeks a blood sample was obtained from these mice via a tail bleed. Upon testing against the analogue-Tg conjugates, as described in section 4.2, antibodies specific to the analogue moiety of the conjugates were detected. Attempts to competitively bind these antibodies with vitamin K<sub>3</sub> were unsuccessful, implying the quinone moiety was not being recognised by the antibody. It was postulated that the 'bridge effect' must again be interfering with the production of antibodies suitable for MCA production.

The analysis of analogue-protein conjugates was attempted by mass spectrometry, however problems with detection of the large molecular ions by electrospray mass spectrometry meant no further information was obtained. Cleavage of the analogue-protein conjugates with a protease and the use of LC-MS is a possible route to overcoming detection problems and thereby obtaining information on the number of analogue molecules attached to each protein, as well as the sites of conjugation. However, time constraints meant this was not possible.

Initial immunological studies using the vitamin K analogues 3.70 and 3.80 encountered problems with differing solubility profiles of the analogues and the proteins. This led to model studies of the peptide coupling process, which suggested that the EDCI coupling was a more effective method than the mixed anhydride coupling for this system. Use of the crude analogue-protein conjugates to inoculate mice led to the isolation of antibodies that recognised the analogue moiety of the conjugate. These antibodies would

not however, competitively bind vitamin K<sub>3</sub> suggesting the quinone core of vitamin K was not being recognised. The design and synthesis of two further analogues of vitamin K (4.5 and 4.7), which do not possess the natural isoprene side chain, was carried out in further attempts to produce an analogue capable of raising monoclonal antibodies towards the quinone core of vitamin K. However, the inoculation of mice with conjugates of 4.5 or 4.6 with BSA failed to produce antibodies suitable for MCA production.

Should MCAs which recognise vitamin K be raised it is recognised that assay development will still be a difficult process due to the lipophilicity of vitamin K and hydrophilicity of antibodies. During the course of an assay the antibody and antigen (vitamin K) must be present in the same phase to allow binding. One possible solution to this problem would be the synthesis of artificial organic soluble antibodies via molecular imprinting.

Molecular imprinting is a technique that involves the production of synthetic examples of molecular recognition.<sup>7</sup> This technique involves the production of an imprinted polymer capable of combining the advantages of synthetic plastics, such as low cost, durability and robustness, with the recognition properties of natural receptors, e.g. antibodies.<sup>7</sup> This is achieved by using elements of the target molecule to create it's own recognition site, by forming a highly cross-linked polymeric matrix around a template, which can be the target molecule itself or a close structural analogue.<sup>7</sup> Non-covalent imprinting has successfully been applied to many compounds of biological importance including amino acid derivatives and peptides,<sup>8</sup>  $\beta$ -blockers,<sup>9</sup> and diazepam.<sup>10</sup> A full investigation of this technique falls beyond the scope of this thesis, but this poses a potential solution to the solubility problems encountered, and therefore a route to a synthetic 'immunoassay' for vitamin K.

## 4.6 REFERENCES FOR CHAPTER FOUR

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## **CHAPTER FIVE**

### **SYNTHESIS OF CoQ ANALOGUES**

## 5.1 INTRODUCTION

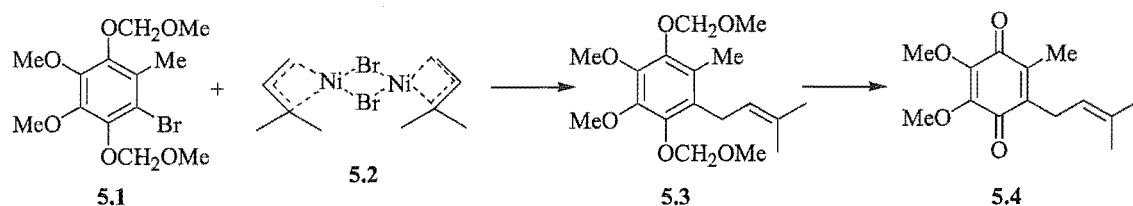
Although structurally related to the K vitamins, coenzyme Q (or ubiquinone) plays a vastly different role in biological systems. Ubiquinones have been shown to function as mobile mediators for electron transfer and proton translocation between redox enzymes in the electron transport chain of mitochondria and bacterial respiratory systems (see chapter 1). Coenzyme Q (CoQ) is required for at least three of the mitochondrial enzymes (complexes I, II and III) as well as enzymes in other parts of the cell. It has been implicated that coenzyme Q-10 (CoQ-10) supplementation is advantageous to a number of disease states including cardiovascular disease, cancer and gum disease, as well as boosting energy and brain power. Due to the remarkable physiological and clinical activity of CoQ-10 there has been much interest in the development of an efficient synthesis of the ubiquinones and analogues of these compounds.

Due to the chemical similarities between the K vitamins and CoQ, it was recognised that analogues of CoQ could be synthesised by a route analogous to that devised for vitamin K (see chapters 3 and 4). Outlined below is a review of current literature procedures for the synthesis of CoQ and its analogues. This is followed by a discussion of the synthesis undertaken towards four side-chain functionalised analogues of CoQ suitable for immunological studies towards the production of an assay for CoQ. Model coupling reactions of one of these analogues to protected amino acids is also discussed.

## 5.2 PREVIOUS WORK ON SYNTHESIS OF COENZYME Q

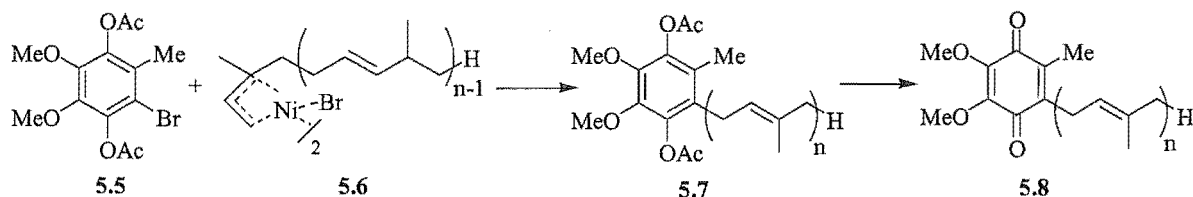
In 1959 Shunk *et al*<sup>1</sup> coupled solanesol, believed to contain 10 isoprene units, to 2,3-dimethoxy-5-methylhydroquinone to give a compound which was found to differ in physical properties from isolated CoQ-10, but was indistinguishable from CoQ-9. From this work solanesol was identified as only containing 9 isoprene units. Shortly thereafter CoQ-10 was successfully synthesised by Ruegg *et al* from decaprenol and 2-methyl-5,6-dimethoxyhydroquinone.<sup>2</sup> Since this time many researchers have endeavoured to increase the efficiency and control of stereochemistry in the synthesis of this important class of compounds.

The use of  $\pi$ -allylnickel halide complexes in the carbon-carbon bond forming step of this synthesis was first studied by Sato *et al* in 1972.<sup>3</sup> The condensation of 6-bromo-2,3-dimethoxy-5-methylhydroquinone bis(methoxymethyl) ether (**5.1**) and 1,1-dimethyl- $\pi$ -allylnickel bromide (**5.2**) in HMPA, followed by hydrolysis of the ether and oxidation, gave CoQ-1 (**5.4**) in 40% yield (scheme 5.1). A similar approach was



### Scheme 5.1

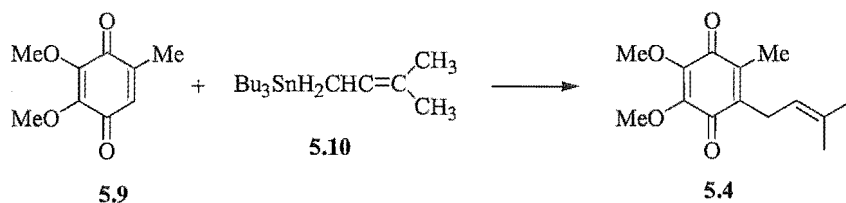
utilised by Hegedus *et al*<sup>4</sup> who synthesised CoQ-1 in one step via the condensation of 2,3-dimethoxy-5-methyl-benzoquinone and 1,1-dimethylallylnickel bromide. This reaction used a 4:1 ratio of quinone:Ni complex and proceeded in 30% yield with respect to consumed quinone, after purification by preparative layer chromatography. This  $\pi$ -allylnickel chemistry was again used by Inoue in 1974 to synthesise CoQ-9 and CoQ-10.<sup>5</sup> Reaction of 1,4-diacetoxy-2-bromo-5,6-dimethoxy-3-methylbenzene (**5.5**) with  $\pi$ -solanesylnickel bromide (**5.6**) (scheme 5.2), followed by removal of the acetate groups and oxidation to the corresponding quinone (**5.8**) gave CoQ-9 in 48% yield. This product was isolated as a mixture with a *cis:trans* ratio of 26:74, which were separable by silica chromatography. Similarly CoQ-10 ( $n=10$ ) was produced in 28% yield, also as a mixture of *cis* and *trans* isomers.<sup>5</sup> The stereoselectivity of this reaction was investigated in various solvents with the highest *trans:cis* ratio occurring with the use of *N,N*-dimethylacetamide, giving 75% *trans* selectivity. Hegedus and Evans studied the mechanism of the reaction of  $\pi$ -allylnickel bromides with quinones, or quinone synthons.<sup>6</sup>



### Scheme 5.2

Maruyama and Naruta in 1978, reported an improved synthesis of CoQ-1 using allyltin reagents.<sup>7</sup> Dimethylallyltributyltin (**5.10**) was reacted with 2,3-dimethoxy-5-

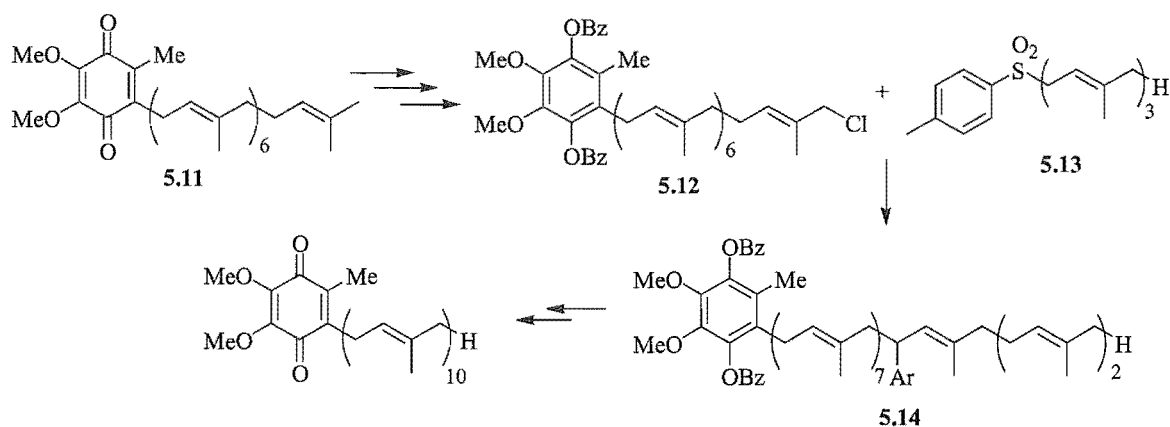
methylbenzoquinone (**5.9**) to give, after chromatography, CoQ-1 (**5.4**) in 75% yield (scheme 5.3). This method had several advantages over earlier syntheses as allylation occurred only at the non-substituted site of the quinone ring, the prenyl group was introduced without isomerisation, and the procedure was shown to be applicable to a range of quinones in good yields (60-90%).<sup>7</sup>



### Scheme 5.3

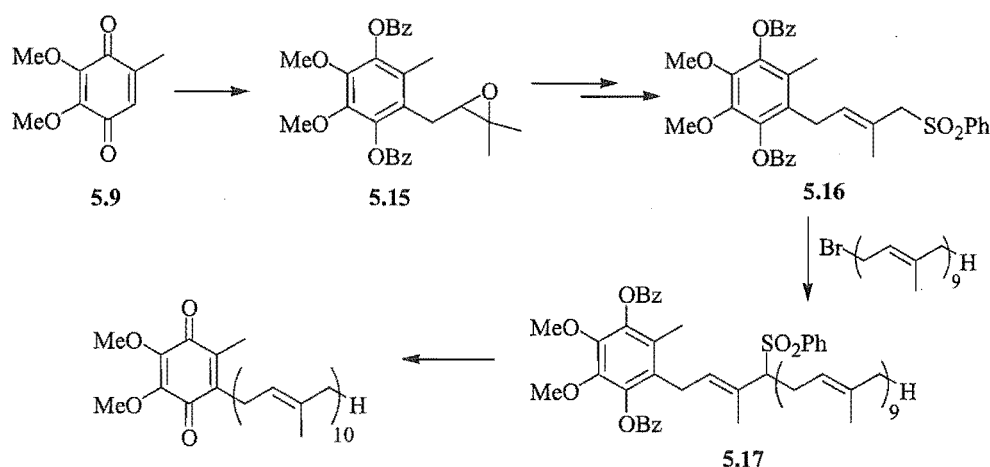
In 1980 Syper *et al*<sup>8</sup> synthesised CoQ-2 using cuprate chemistry. The formation of the diaryl cuprate of 1-methyl-2,3,4,5-tetramethoxybenzene using CuBr, followed by reaction with geranyl bromide afforded CoQ-2 in 66% yield after oxidative deprotection of the quinone.

Due to the high expense and limited availability of the decaprenyl unit for the synthesis of CoQ-10, many researchers elected to build the polyprenyl chain from simpler monomer units which were more readily available. The reaction of an allyl halide with a *p*-tolylsulfonate was a popular approach. The first synthesis of CoQ using this approach was reported by Terao *et al*<sup>9</sup> in 1978, where side-chain elongation was used to convert CoQ-7 to CoQ-10 (scheme 5.4). Through a series of functional group interconversions the terminal double bond of the prenyl side chain of CoQ-7 (**5.11**) was converted to an allyl chloride (**5.12**). This could then be reacted with *trans, trans* *p*-tolylsulfonylfarnesene (**5.13**) to give a protected CoQ-10 analogue (**5.14**) with complete retention of stereochemistry. Deprotection and oxidation gave CoQ-10 in 33% yield overall.



Scheme 5.4

A year later Terao *et al* utilised the same chemistry to synthesise CoQ-10 from CoQ-0.<sup>10</sup> In this case however, the benzylic core was functionalised with a single isoprene unit bearing a phenylsulfonate group (5.16), and reacted with solanesyl bromide (scheme 5.5). When followed by removal of the benzyl and phenylsulfonyl groups, and oxidation of the resulting hydroquinone this gave CoQ-10 in 49% yield.<sup>10</sup>



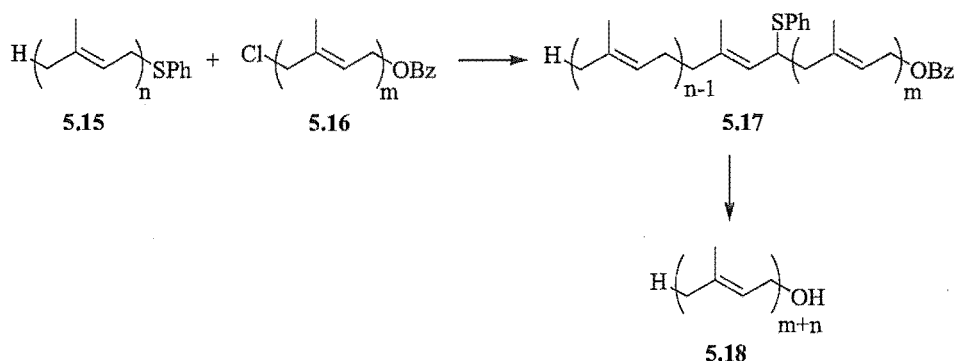
Scheme 5.5

Since this work several groups have reported modifications to this chemistry in an attempt to increase yields, and make these reactions more amenable to larger scale syntheses. In 1982 Fujita *et al*<sup>11</sup> presented an improved synthesis of the sulfone functionalised benzene intermediate (5.16) in only two steps from 2,3-dimethoxy-5-methylhydroquinone. The same year Sato *et al*<sup>12</sup> proposed a slightly different approach to CoQ-10 using the same chemistry outlined above. Coupling of the Grignard reagent of a protected quinone core with isoprene epoxide in the presence of CuCl afforded the corresponding *trans* allylic alcohol in 77% yield. This alcohol was then converted to the bromide and coupled with solanesyl *p*-tolyl sulfone. After removal of the sulfone groups and deprotection of the quinone this afforded CoQ-10.



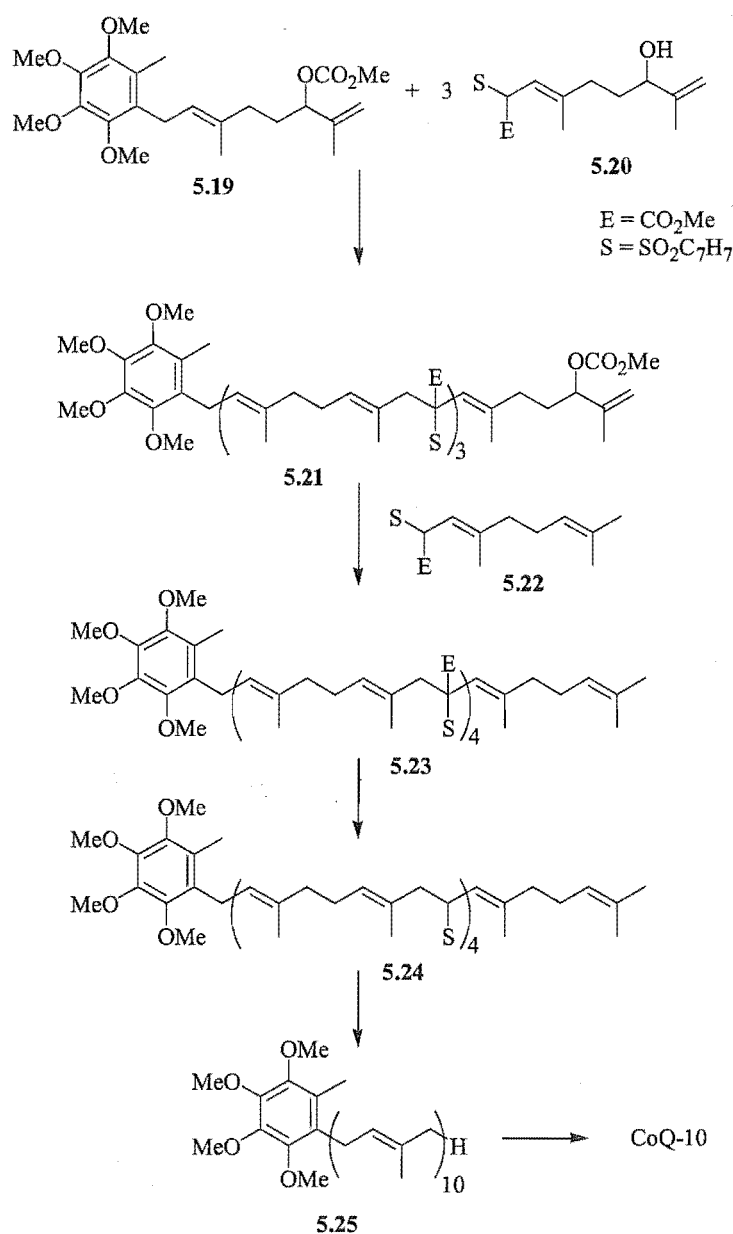
In 1986 Mohri *et al*<sup>13</sup> presented an investigation of the desulfonylation reaction in an effort to overcome the production of a small amount of the  $\Delta^3$  alkene product. By choosing a synthon which placed the sulfone at the 5-position of the side chain, instead of the 4-position, complete stereoselectivity for the  $\Delta^2$  alkene was observed, but 5% of the *cis* isomer at the  $\Delta^6$  position was isolated.

Naruta<sup>14</sup> in 1980, synthesised an all-*trans* decaprenyl side chain from simpler prenyl units using a similar approach (scheme 5.5). A phenylsulfonylprenyl unit (5.15) was reacted with an allyl chloride prenyl unit (5.16) to effect chain elongation. Once the desired chain length was achieved, this was converted to the corresponding trimethylpolyprenylstannane, which was coupled to a quinone core using 3 equivalents of  $\text{BF}_3 \cdot \text{OEt}_2$ . This method was used to synthesise CoQ-3 to CoQ-9 in yields of 40 to 90% with a *trans:cis* ratio of 98:2. CoQ-10 was also synthesised in this way, in 51% yield with a *trans:cis* ratio of 86:14. The reason for the lower stereoselectivity in this case is not discussed.<sup>14</sup>



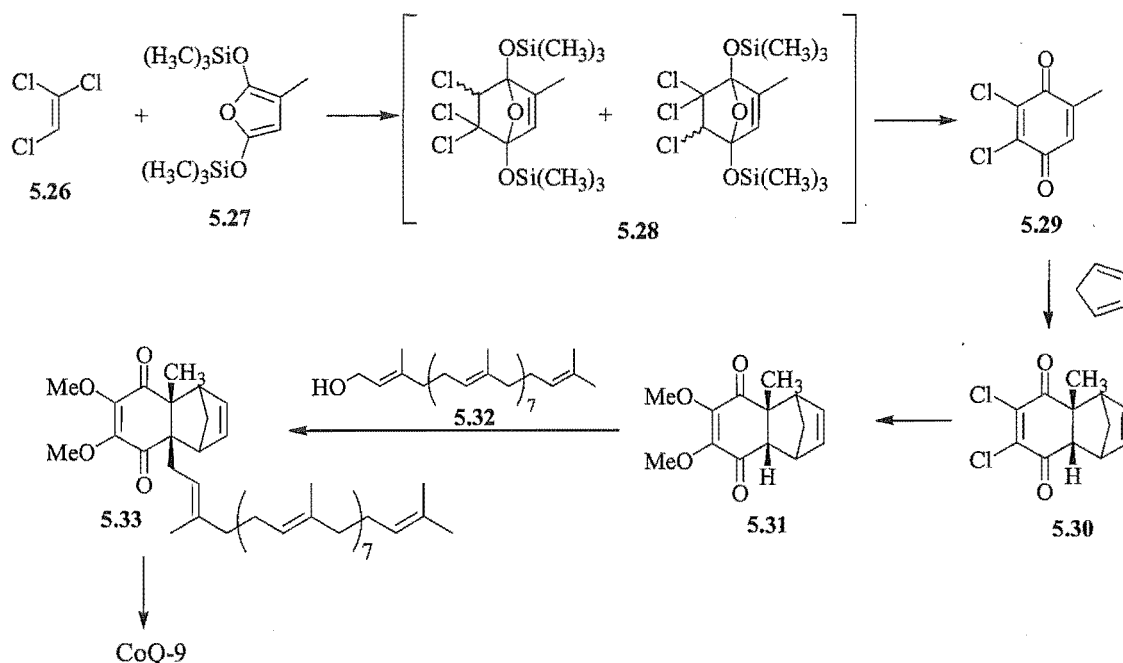
**Scheme 5.5**

This strategy of systematically building the polyprenyl side chain from simpler units was also employed by Eren *et al* (scheme 5.6).<sup>15</sup> They designed an oligomerisation approach utilising monoterpene monomers derived from geraniol, and Pd(0) coupling techniques. Once a chain of the desired length had been synthesised the substituents remaining from the coupling process were removed collectively. The methyl carbonate groups were removed initially with *p*-aminothiophenol and cesium carbonate, followed by removal of the sulfonyl groups with  $\text{Pd}(\text{dppp})\text{Cl}_2$  and lithium triethylborohydride. Final deprotection of the quinone with CAN afforded CoQ-4 in 60% yield from 5.19 and CoQ-10 in 20% yield from 5.19.<sup>15</sup>



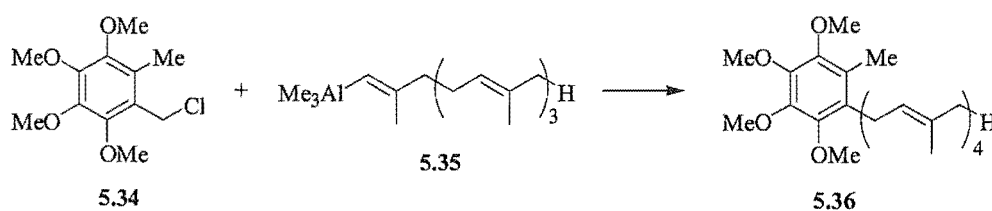
Scheme 5.6

Another approach used in the synthesis of ubiquinones is based on Diels-Alder chemistry (scheme 5.7).<sup>16</sup> In this strategy the quinone moiety is synthesised via a Diels-Alder reaction between 1,1,2-trichloroethene (5.26) and 2,5-bis[(trimethylsilyl)oxy]-3-methylfuran (5.27). Methanolysis of the Diels-Alder adduct (5.28) to give 5.29 was followed by reaction with cyclopentadiene and substitution of the chloride groups for methoxy groups to give a diketone intermediate (5.31). This intermediate is readily alkylated with the desired polyprenol (5.32) using a strong base to give 5.33. Finally a retro-Diels-Alder reaction reveals the corresponding CoQ in a yield of 26% for both CoQ-5 and CoQ-9 from 5.29.<sup>16</sup>



Scheme 5.7

The coupling reaction of a polyprenyl side chain and protected core has more recently been investigated by Lisphutz *et al.*<sup>17</sup> The core compound used in this case is a benzylic halide (5.34) which is reacted with vinylalane 5.35 in the presence of 0.5 mol % Ni(0) catalyst at room temperature for 15 minutes (scheme 5.8), to afford 5.36 in 87% yield. This is an improvement over the previously used methods of copper catalysed Grignard coupling, which didn't give complete retention of stereochemistry,<sup>18</sup> and Pd(0) based couplings which are more expensive, employ harsh reaction conditions and require longer reaction times, while proceeding in lower yields.<sup>15</sup>



Scheme 5.8

A small amount of work has also gone into producing various analogues of coenzyme Q including fluorescently<sup>19</sup> and isotopically labelled analogues.<sup>20</sup> The study of protein-coenzyme Q analogue interactions by He *et al.*<sup>21,22</sup> has also led to the synthesis of many analogues of CoQ, including compounds alkylated at the 5-position<sup>21</sup> and ethoxy- derivatives of the 2- and 3- positions.<sup>22</sup> Analogues with side chains containing various levels of unsaturation have also been synthesised in an

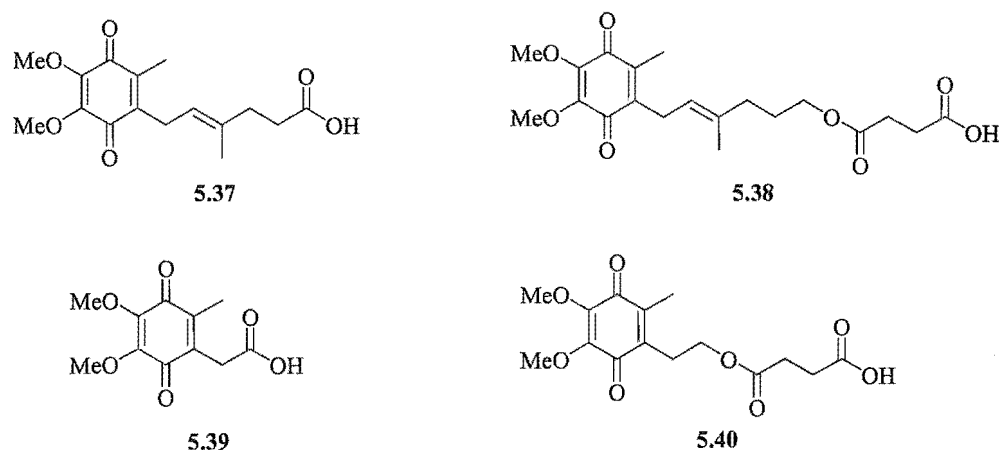
effort to establish the role of the isoprene chain in the interaction between respiratory enzymes and CoQ.<sup>23</sup>

Of more interest for our work are the limited reports of the synthesis of side-chain functionalised analogues of CoQ. In 1978 Terao *et al* produced a series of such analogues in the process of functionalising CoQ-7 for chain elongation.<sup>9</sup> These included such functionalities as bromohydrins, epoxides, diols, aldehydes, allyl acetates and allyl alcohols. Some years later the same group prepared a series of quinone acid, amide, alcohol and methylketone derivatives.<sup>24</sup> These compounds were synthesised via a series of functional group interconversions of CoQ compounds with varying chain length. The synthetic route used is the same as that described previously (scheme 3.14) for the corresponding synthesis of vitamin K analogues.

The synthetic route outlined below, to the production of side-chain functionalised analogues of CoQ (5.37 and 5.38) is similar to that used by Terao *et al*<sup>24</sup>. However, as was the case with vitamin K<sub>2</sub>, the scheme has been shortened to remove unnecessary chain elongation steps. As the chemistry of vitamin K and CoQ are so closely related the same synthetic route was applied to the production of analogues of CoQ as outlined in sections 3.5 and 4.4 for the synthesis of vitamin K analogues.

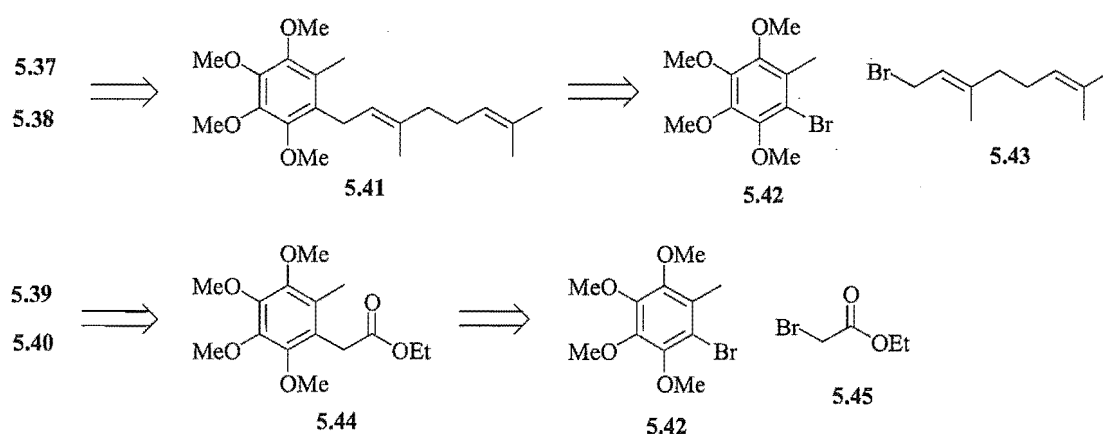
### 5.3 SYNTHESIS OF COENZYME Q ANALOGUES

As discussed for the case of vitamin K earlier in this thesis, the synthesis of analogues of CoQ was undertaken with emphasis on the production of an analogue suitable for conjugation to a carrier protein (see section 3.1). For this reason a carboxylic acid functionality at the side chain terminus of the target analogue was chosen to allow amide bond formation with the amine group of a lysine side chain on the surface of a potential carrier protein. Upon successful synthesis of such an analogue, and conjugation to a carrier protein, this may be used to inoculate mice, stimulating the production of antibodies for use in the preparation of monoclonal antibodies (see chapter 4 for details). The synthetic route adopted is a parallel synthesis to that described earlier for the synthesis for vitamin K analogues. The initial target compounds designed are shown in figure 5.1.



**Figure 5.1:** Structures of desired coenzyme Q analogues

The retrosynthetic analysis of these target analogues is shown in figure 5.2. All of these compounds arise from the coupling of a protected quinone core (5.42) and a side chain moiety (5.43 or 5.45). The first step therefore, in the synthesis of these analogues, is the production of 1-bromo-2-methyl-3,4,5,6-tetramethoxybenzene (5.42), the protected benzoquinone core.

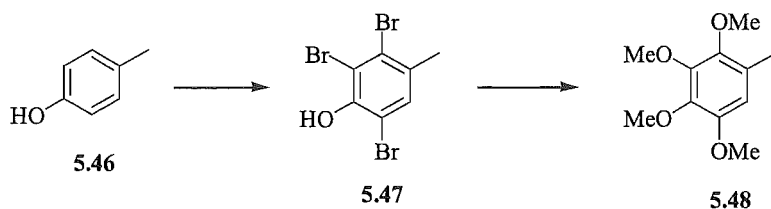


**Figure 5.2:** Retrosynthetic analysis of CoQ analogues

### 5.3.1 SYNTHESIS OF PROTECTED CORE MOEITY

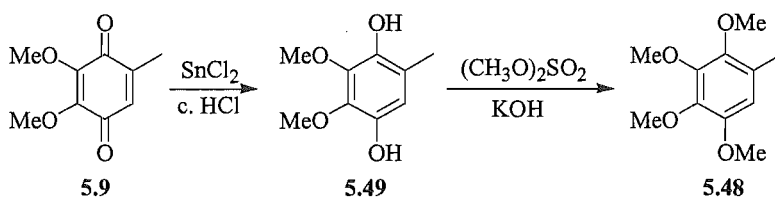
The protected quinone core (5.42) is readily synthesised via bromination of 1-methyl-2,3,4,5-tetramethoxybenzene (5.48). Upon reviewing the literature, it was found that many groups had synthesised 5.48 via complicated multistep procedures from highly functionalised aromatic starting materials such as vanilline,<sup>25</sup> and pyrogallol.<sup>8</sup> However, Keinan and Eren<sup>26</sup> published a convenient two step synthesis

of 1-methyl-2,3,4,5-tetramethoxybenzene (**5.48**) from the readily available starting material *p*-cresol (**5.56**) (scheme 5.9), reporting an overall yield of 71%.



**Scheme 5.9**

Tribromination of *p*-cresol (**5.46**) with bromine in chloroform afforded 2,3,6-tribromo-4-methylphenol (**5.47**) as white needles in 61% yield after recrystallisation from petroleum ether. The second step in the synthesis, copper-catalysed methoxylation of **5.47**, followed by methylation with dimethyl sulfate to yield **5.48** is a one-pot process.<sup>26</sup> Following the literature procedure this yielded a crude product, which was purified using radial chromatography, into three fractions. Identification of these fractions proved difficult by <sup>1</sup>H NMR analysis as all protons in the various products were in similar chemical environments. For this reason all characterisation was carried out by mass spectrometry. The first fraction contained a product which still possessed two bromine atoms, the second fraction was a mixture of **5.48** and a monobrominated product, and the third fraction contained a mixture of minor by-products which were not identified. Despite this reaction being attempted several times, yields of the desired product were poor and isolation by chromatography proved difficult, even though a 94% yield without need for purification was reported in the literature.<sup>26</sup> Due to the problems encountered, a new synthetic route to **5.48** was developed (scheme 5.10). This synthesis involved the protection of 2,3-dimethoxy-5-methyl-1,4-benzoquinone (CoQ-0) (**5.9**) in a process analogous to that used in the protection of vitamin K<sub>3</sub> (see section 3.4.1).

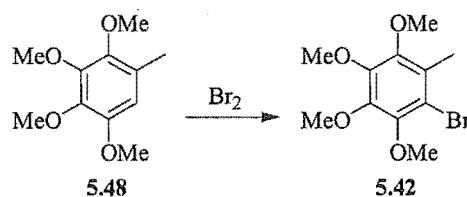


**Scheme 5.10**

Reaction of **5.9** with stannous chloride and concentrated hydrochloric acid, followed by extraction into ethyl acetate afforded an off white solid identified as **5.49** by <sup>1</sup>H NMR analysis. This isolated product was directly methylated to **5.48** in order

order to minimise any reoxidation to the quinone. The hydroquinone mixture was suspended in dimethylsulfate and potassium hydroxide was added portionwise. Reaction at 80 °C for one hour followed by extraction, yielded **5.48** as a pale yellow oil (85% yield over two steps).

The final step in the preparation of a core compound suitable for analogue production is the bromination of 1-methyl-2,3,4,5-tetramethoxybenzene (**5.48**). This was carried out following the procedure of Syper *et al.*<sup>8</sup> Reaction of **5.48** with bromine for 10 minutes afforded 1-bromo-2-methyl-3,4,5,6-tetramethoxybenzene (**5.42**) as a yellow oil in 84% yield. The isolated product was found to be clean by <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy, and could be identified by the loss of any proton resonance at 6.44 ppm corresponding to the aromatic proton of **5.48**. The singlet of the methyl ether protons also shifted downfield from 2.22 ppm for **5.48** to 2.30 ppm for **5.42**. This isolated compound was used in the synthesis of coenzyme Q analogues without further purification.



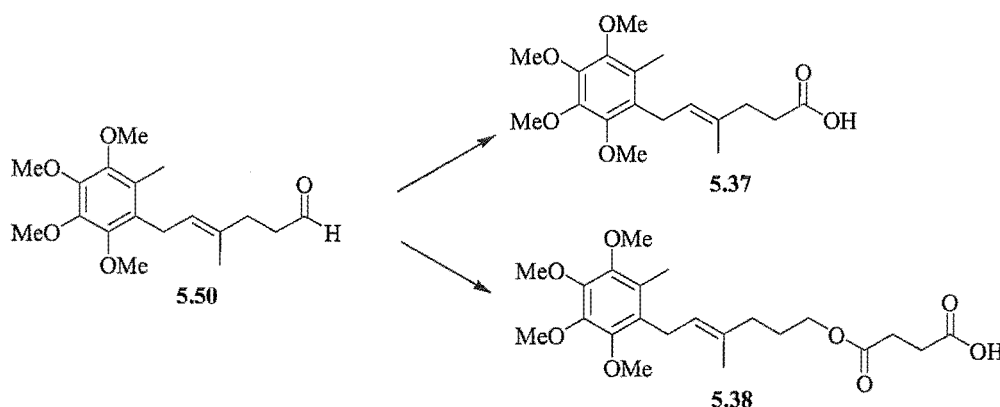
**Scheme 5.11**

A new and efficient synthesis of 1-methyl-2,3,4,5-tetramethoxybenzene (**5.42**) from 2-methyl-5,6-dimethoxy-1,4-benzoquinone (**5.9**) was developed. This involved the reduction of CoQ-0 (**5.9**) with stannous chloride and concentrated hydrochloric acid to give the corresponding hydroquinone (**5.49**). This hydroquinone was directly methylated with dimethylsulfate and KOH to afford **5.48**. Using the method of Syper *et al.*,<sup>8</sup> **5.48** was brominated to give the desired protected benzoquinone core **5.42**, for use in the synthesis of coenzyme Q analogues.

### 5.3.2 SYNTHESIS OF COENZYME Q ANALOGUES

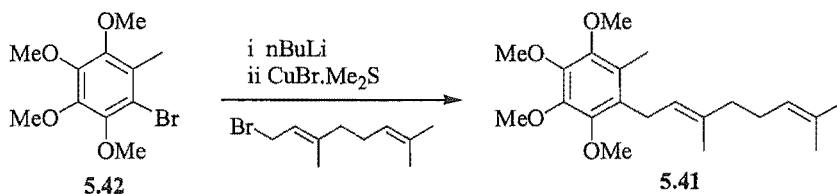
The synthetic route described below for the preparation of coenzyme Q analogues is analogous to that used in the preparation of vitamin K analogues (see sections 3.5 and 4.4). The target analogues (see figure 5.1), were designed based upon the second series of vitamin K analogues produced (**4.5** and **4.7**) (see section 4.4).

Analogues **5.37** and **5.38** were both synthesised from the common intermediate 6-(2-methyl-3,4,5,6-tetramethoxyphenyl)-4-methylhex-4-enal (**5.50**) (see figure 5.3).



**Figure 5.3:** Intermediate aldehyde (**5.50**), used for the synthesis of **5.37** and **5.38**

The first step in the synthesis of **5.50** is the coupling of 1-bromo-2-methyl-3,4,5,6-tetramethoxybenzene (**5.42**) with geranyl bromide.<sup>8</sup> The diaryl cuprate of **5.42** was formed via treatment with *n*BuLi and copper bromide dimethylsulfide, and this was then reacted with geranyl bromide. Purification of the crude product by radial chromatography gave **5.41** as a yellow oil in 50% yield. This yield was lower than expected based on the preparation of **3.67** (see section 3.4.3), and literature results of 76% yield.<sup>8</sup> This may be due to the need for recrystallisation of the copper bromide dimethylsulfide complex, which loses dimethylsulfide over time leading to incorrect stoichiometry of the reaction. Analysis of the isolated product (**5.41**) by <sup>1</sup>H NMR spectroscopy showed evidence of the presence of trace amounts of the terminal alkene, arising from attack at the 3 position of the alkyl bromide. This was suggested by the presence of two small multiplets at 4.88 and 6.27 ppm, indicative of the proton resonances of a terminal alkene.



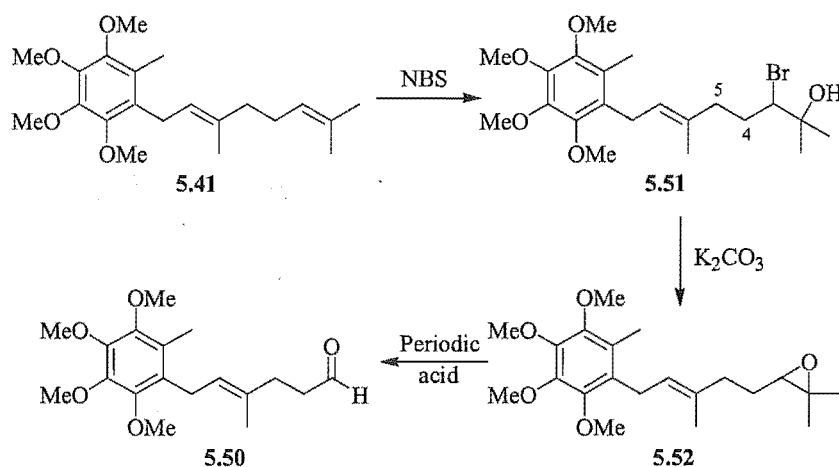
### Scheme 5.12

Treatment of **5.41** with *N*-bromosuccinimide, followed by work up and purification by radial chromatography afforded the bromohydrin (**5.51**) as a yellow oil in 65% yield (scheme 5.13). Upon analysis by <sup>1</sup>H NMR spectroscopy the two C-4 proton resonances were multiplets at 1.79 and 1.98 ppm, while the C-5 proton



resonances were two multiplets at 2.09 and 2.35 ppm. Each of these four protons is in a unique chemical environment, and hence have a different chemical shift, due to the presence of a stereogenic centre at C-3, which makes the protons at C-4 and C-5 diastereotopic.

Reaction of the bromohydrin (**5.51**) with potassium carbonate gave the corresponding epoxide (**5.52**) in 78% yield (scheme 5.13). Analysis of the isolated product by  $^1\text{H}$  NMR spectroscopy showed the absence of a doublet at 3.95 ppm corresponding to the C-6 proton of **5.51**, and the presence of the corresponding proton resonance of the epoxide as a triplet at 2.67 ppm. This product was clean by both  $^1\text{H}$  and  $^{13}\text{C}$  NMR analysis, and was used without further purification.

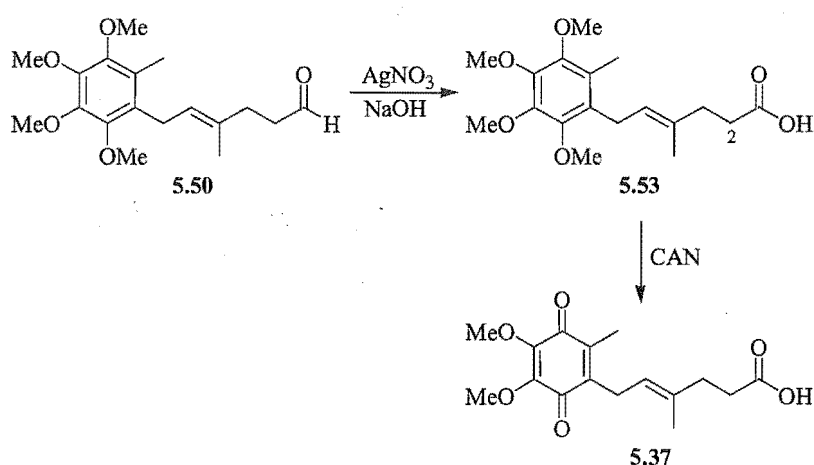


### Scheme 5.13

Reaction of the epoxide (**5.52**) with periodic acid yielded, after work up, a yellow oil (85%), which was identified by  $^1\text{H}$  NMR analysis to be aldehyde **5.50** (scheme 5.13). This was confirmed by the presence of a singlet in the  $^1\text{H}$  NMR spectrum at 9.74 ppm, and a peak in the  $^{13}\text{C}$  NMR spectrum at 202.47 ppm, both corresponding to the aldehyde functionality of **5.50**. This aldehyde (**5.50**) was used in the synthesis of **5.37** and **5.38** without further purification.

Target analogue **5.37** was synthesised from the aldehyde (**5.50**) in two steps (scheme 5.14). The oxidation of **5.50** to the carboxylic acid **5.53** was achieved with silver nitrate and sodium hydroxide in ethanol, and afforded after work up, **5.53** as a yellow oil in 78% yield. NMR spectroscopy of the carboxylic acid showed few differences from the spectra obtained for **5.50**. The  $^1\text{H}$  NMR spectrum of **5.53** no longer possessed a peak at 9.74 ppm, and showed an upfield shift from 2.52 to 2.45

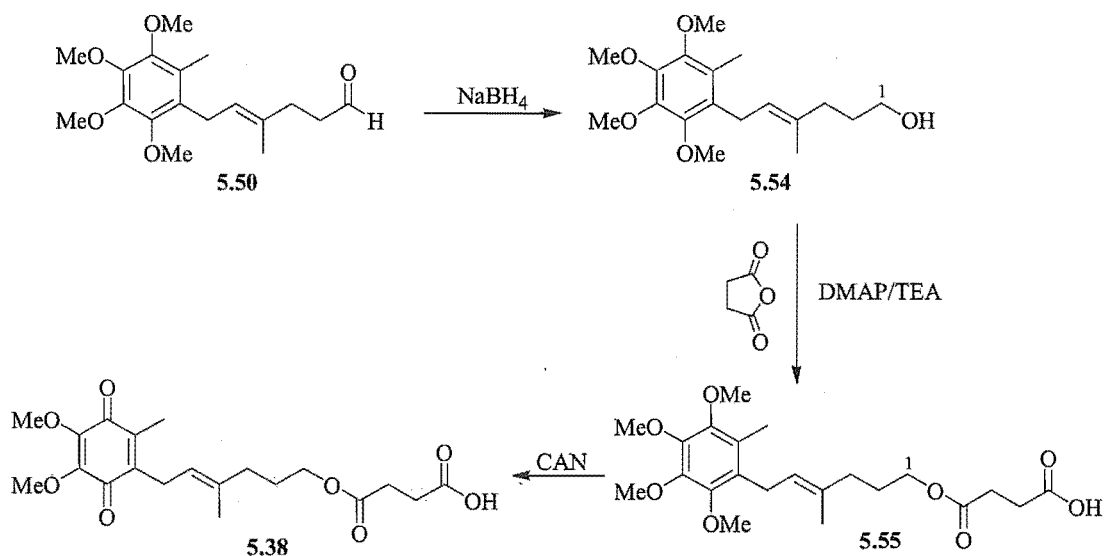
ppm for the C-2 methylene proton resonance. In the  $^{13}\text{C}$  NMR spectrum of **5.53** the aldehyde signal at 202.47 ppm, was replaced with a peak at 178.47 ppm corresponding to the carbon of the carboxylic acid functionality. This carboxylic acid (**5.53**) was used without any further purification. Deprotection of the quinone core with CAN proceeded in 55% yield to give target analogue **5.37** as a yellow oil after purification by radial chromatography. The disappearance of the methyl ether resonances at 3.77 and 3.78 ppm in the proton NMR spectrum of **5.37**, together with the appearance of two peaks at 183.79 and 184.60 ppm in the corresponding  $^{13}\text{C}$  NMR spectrum characteristic of the quinone, confirmed that deprotection to the 1,4-benzoquinone had occurred.



**Scheme 5.14**

The synthesis of target analogue **5.38** from aldehyde **5.50** is shown in scheme 5.15. Reduction of the aldehyde (**5.50**) with sodium borohydride afforded the corresponding alcohol (**5.54**) as a pale yellow oil in 74% yield. Analysis by  $^1\text{H}$  NMR spectroscopy showed no resonance corresponding to the aldehyde proton at 9.74 ppm, and the presence of the C-1 methylene proton resonance at 3.59 ppm, confirming that reduction to the alcohol had occurred. This alcohol was reacted with succinic anhydride in the presence of triethylamine and *N,N*-dimethylaminopyridine for 18 hours. Following work up, the crude product was purified by radial chromatography to give the succinic ester (**5.55**) as a pale yellow oil (82% yield). The downfield shift of the C-1 methylene resonance from 3.59 ppm for **5.54** to 4.05 ppm for **5.55**, is explained by the conversion of the adjacent functionality from an alcohol to an ester. The presence of two multiplets in the  $^1\text{H}$  NMR spectrum of **5.55** at 2.59 and 2.65 ppm, and  $^{13}\text{C}$  NMR signals at 172.22 and 176.96 ppm corresponding to the succinyl ester moiety, also confirmed that esterification had occurred.

Deprotection with CAN proceeded in 80% yield to afford target analogue **5.38** as an orange oil after purification by radial chromatography. As with **5.37**, the disappearance of the methyl ether proton resonances at 3.78 and 3.79 ppm in the  $^1\text{H}$  NMR spectrum, and the appearance of  $^{13}\text{C}$  NMR signals at 183.93 and 184.74 ppm, confirmed oxidation to the 1,4-benzoquinone was successful.

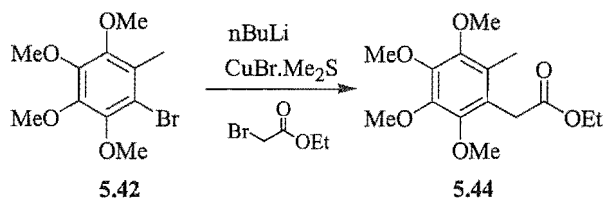


**Scheme 5.15**

The retrosynthetic analysis for target analogue **5.39** was shown in figure 5.2. The first step in the synthesis of this analogue is the coupling of the 1-bromo-2-methyl-3,4,5,6-tetramethoxybenzene core (**5.42**) and ethyl bromoacetate (scheme 5.16).

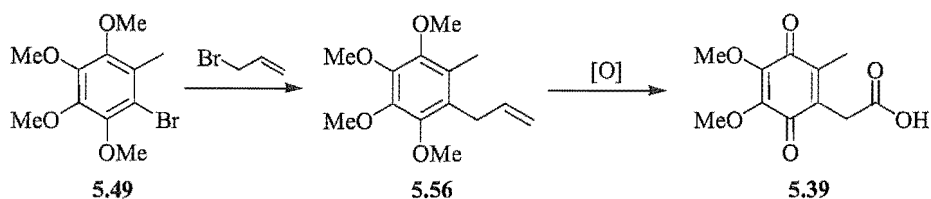
Reaction of **5.42** and ethyl bromoacetate under standard cuprate coupling conditions<sup>8</sup> gave a crude reaction mixture which was purified by radial chromatography to give three fractions. Analysis by  $^1\text{H}$  NMR spectroscopy of these fractions showed fraction one contained a complex mixture of minor by-products, fraction two contained 1-methyl-2,3,4,5-tetramethoxybenzene, and the desired product was isolated in the third fraction as a yellow oil in only 7% yield.  $^1\text{H}$  NMR analysis of this product showed an upfield shift of the aromatic methyl resonance from 2.31 ppm for **5.42** to 2.09 ppm for **5.44**. The other  $^1\text{H}$  NMR signal indicative of reaction was the methylene resonance at 3.85 ppm in ethyl bromoacetate, and 3.51 ppm in the coupled product **5.44**. Although this reaction was repeated several times no increase in this yield was observed. A low yield when using methyl bromoacetate in cuprate coupling reactions, in comparison with alkyl bromides, has been previously documented.<sup>27</sup> A drop in yield from the coupling reaction of **3.11** with geranyl

bromide to that of **3.11** with ethyl bromoacetate was also observed in the synthesis of vitamin K analogues as discussed earlier (see chapter 4).



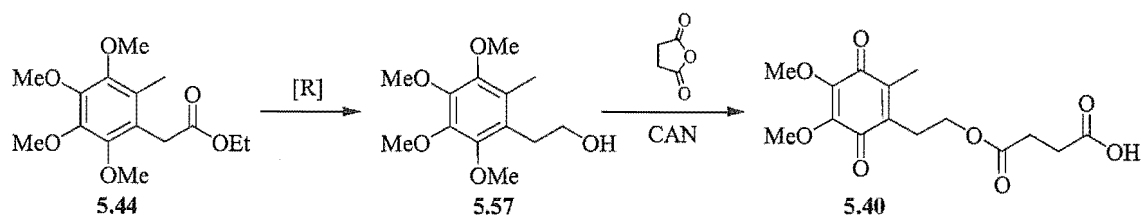
**Scheme 5.16**

One alternative synthetic route to target **5.39** is outlined in scheme 5.17. This approach eliminates the low yielding cuprate coupling with ethyl bromoacetate, and replaces it with the use of allyl bromide for this coupling reaction, which has been documented to proceed in high yield.<sup>27</sup> Oxidation and subsequent deprotection of the coupled product **5.56**, would give the desired analogue **5.39**. Due to lack of time this approach was not attempted, but this is a good starting point for future work in this area. Many other transition metal based coupling procedures have also been reported for the synthesis of coenzyme Q and related compounds, and these could also be investigated.



**Scheme 5.17**

As the synthesis of analogue **5.40** also requires intermediate **5.44** (scheme 5.16), synthesis of **5.40** was not completed due to time constraints. Analogue **5.40**, however, could be synthesised from **5.44** via the synthetic route outlined in scheme 5.18. This chemistry has been investigated for the synthesis of vitamin K analogues, and it is expected to proceed smoothly.



**Scheme 5.18**

Synthesis of the aldehyde intermediate (**5.50**), for use in the preparation of CoQ analogues **5.37** and **5.38** proceeded via functionalisation of the terminal alkene of **5.41**. Although the method used for the bromohydrin formation and epoxidation is reported in the literature,<sup>24</sup> data on the bromohydrin (**5.51**) was not reported. The cleavage of epoxide **5.52** gave rise to the aldehyde **5.50**, which has not previously been reported.

A new synthesis to analogue **5.37** has been developed which is shorter and more efficient than that previously presented by Terao *et al.*<sup>24</sup> By oxidation of the intermediate aldehyde **5.50**, and subsequent oxidative deprotection to the analogue **5.37**, rather than claisen rearrangement of a corresponding allylic alcohol we have reduces the number of steps and amount of purification required for this synthesis.

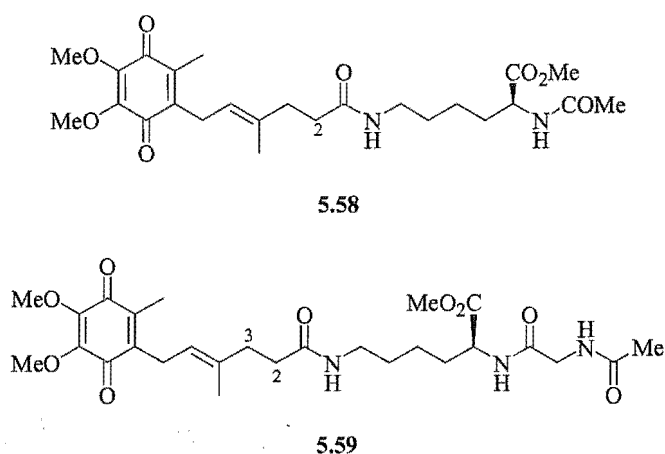
The synthesis of analogue **5.38** has not previously been reported. This analogue was prepared in three steps from the intermediate aldehyde **5.50**. Reduction of the aldehyde (**5.50**) to the corresponding alcohol, followed by esterification with succinic anhydride and oxidative deprotection afforded **5.38** which was fully characterised by one and two-dimensional NMR analysis and mass spectrometry.

Due to the low yields observed in the cuprate coupling reaction of **5.49** with ethyl bromoacetate, synthesis analogues **5.39** and **5.40** was not achieved. Another route to analogue **5.39** utilising the cuprate coupling of **5.42** with allylic bromide, which has been previously studied, has been presented. The use of other transition metals could also be investigated to effect the coupling of **5.42** and ethyl bromoacetate. Although time constraints meant these alternatives could not be investigated there is a good basis for future synthetic studies in this area.

## 5.4 COUPLING OF ANALOGUES TO DIPEPTIDES

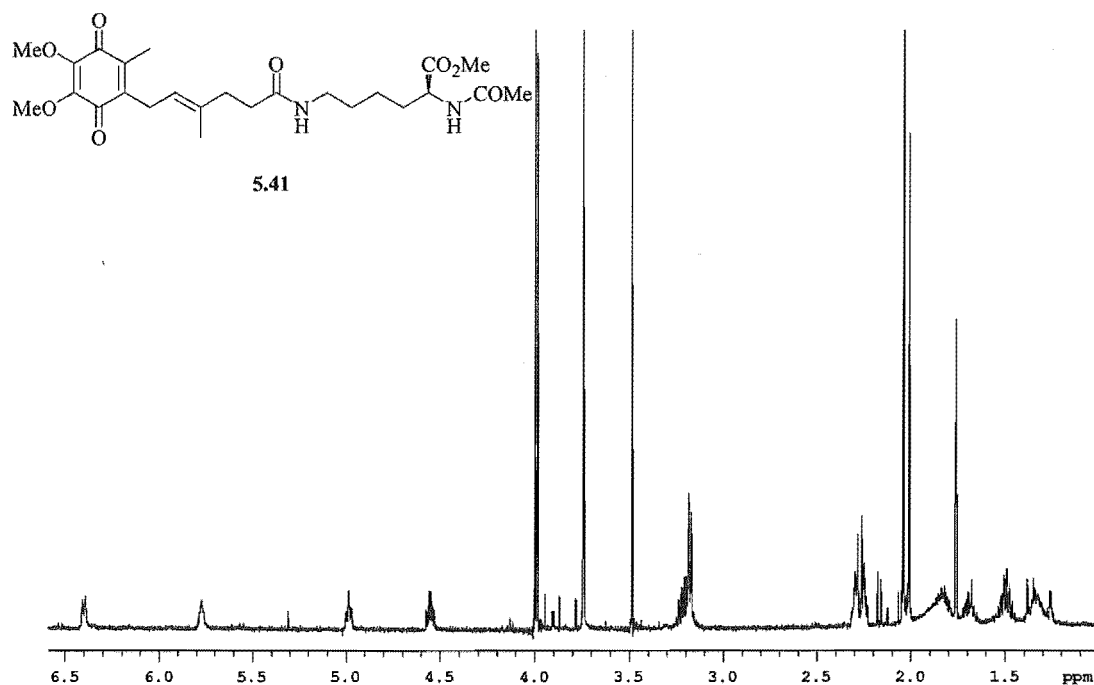
Having successfully completed the synthesis of two analogues of coenzyme Q (**5.37** and **5.38**), **5.37** was used in model peptide coupling reactions. The conjugation of analogues to a carrier protein for immunological study, will occur via the reaction of amino group of the side chain of lysine residues on the peptide with the carboxylic acid functionality of the analogue. For this reason, in model studies, protected lysine and a protected lysine-glycine dipeptide were used.

The peptide coupling method chosen for the reaction of **5.37** with *N*- $\alpha$ -acetyl-lysine methyl ester and *N*-acetyl-glycine-lysine methyl ester was that mediated by EDCl. This coupling procedure was chosen over the other possible coupling methods in light of solubility and purification problems observed in the analogous couplings with vitamin K analogues (see chapter 4). The products synthesised in this manner are shown in figure 5.4.



**Figure 5.4:** Structures of CoQ analogue-amino acid conjugates

The coupled products were analysed and characterised by <sup>1</sup>H, <sup>13</sup>C, and two-dimensional NMR techniques, as well as by mass spectroscopy. Upon analysis of the <sup>1</sup>H NMR spectrum of conjugate **5.58** several features confirmed the presence of the desired product (see figure 5.5). The presence of the amide resonances at 5.77 and 6.40 ppm in the <sup>1</sup>H NMR spectrum, and a multiplet at 4.55 ppm corresponding to the  $\alpha$ -proton of the lysine residue confirmed successful conjugation. The side chain methylene proton resonances of lysine were observed as a series of multiplets at 1.35 (2H), 1.50 (2H), 1.69 (1H), 1.82 (1H), and 3.21 (2H) ppm. The upfield shift of the methylene protons at C-2, from 2.43 ppm in **5.37** to 2.29 ppm in the conjugate, indicates the change in functionality from an acid to an amide adjacent to this site.



**Figure 5.5:**  $^1\text{H}$  NMR spectrum of conjugate 5.41

Similarly, analysis of conjugate 5.42 by  $^1\text{H}$  NMR spectroscopy gave similar results. The  $^1\text{H}$  NMR spectrum of 5.42 contained three resonances at 6.03, 6.74, and 7.08 ppm indicating the presence of three amide protons. The  $\alpha$  proton resonance of lysine was observed as a multiplet at 4.53 ppm, while the side chain methylene proton resonances of the lysine residue were observed as a series of multiplets at 1.34, 1.46, 1.71, 1.85, and 3.23 ppm. The  $\alpha$  proton resonance of the glycine residue was observed as two doublet of doublets at 3.91 and 4.01 ppm. In this conjugate the methylene protons of C-2 and C-3 are no longer observed as two signals, but a single multiplet at 2.28 ppm.

During this study two analogues of CoQ were successfully synthesised. A shorter more efficient route to the previously known analogue 5.37 is presented, while 5.38 has been synthesised as a novel compound via a route analogous to that described for the synthesis of vitamin K analogues is chapter 3. Synthesis of analogues 5.39 and 5.40 were not completed due to time constraints and low yields of the cuprate coupling reaction between 5.42 and ethyl bromoacetate. Further study into the presented alternative routes to these analogues is required.

The analogues of coenzyme Q synthesised in this study are suitable for use in studies towards the feasibility of an immunoassay for coenzyme Q levels in biological tissues. As such an assay is not currently available, and coenzyme Q has been shown

to be important in many diseased states, this could be important for use in research and routine assessment in medical areas.



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## **CHAPTER SIX**

### **EXPERIMENTAL**

## 6.1 GENERAL PROCEDURES

### Nuclear Magnetic Resonance

Proton detected NMR spectra were obtained on either a Varian Unity 300 spectrometer operating at 300 MHz, or an Inova 500 spectrometer operating at 500 MHz. Carbon detected NMR spectra were obtained on a Varian XL300 spectrometer or a Varian Unity 300 spectrometer both operating at 75MHz. All spectra were obtained at 23 °C. Other two-dimensional NMR experiments used include HSQC, HMBC and CIGAR experiments, which were all obtained on the Inova 500 spectrometer at 500 MHz. Chemical shifts are reported in parts per million (ppm), on the  $\delta$  scale, and are referenced to the appropriate solvent peaks:  $\text{CDCl}_3$  referenced to  $(\text{CH}_3)_4\text{Si}$  at  $\delta$  0 ppm for  $^1\text{H}$  spectra, and  $\text{CDCl}_3$  at  $\delta$  77.0 ppm for  $^{13}\text{C}$  spectra. All  $^{13}\text{C}$  NMR spectra were obtained with a delay ( $D_1$ ) of 1 s.  $^1\text{H}$  NMR spectra were assigned by comparison, with all assignments verified by 2-D NMR experiments.

### Mass Spectrometry

Mass spectrometry was performed by Electron Impact (EI) at 4 kV using a Kratos MS80 Mass Spectrometer. Where softer ionisation was required the technique of Fast Atom Bombardment (FAB) was used. All amino acid conjugates were analysed by electrospray mass spectrometry using a Micromass LCT spectrometer.

### IR Spectroscopy

IR spectra were obtained using a Shimadzu 8201PC series FTIR. All oils were analysed neat on KBr disks, and solids were pressed into a KBr disk for analysis.

### Melting Points

Melting points were taken on an electrothermal apparatus and are uncorrected.

### Reagents and Solvents

Unless otherwise stated all reactions were carried out in oven-dried, or flame-dried glassware. Analytical thin layer chromatography (TLC) was conducted on plastic-backed Merck Keiselgel KG60F<sub>254</sub> silica plates, and visualised using short wavelength ultraviolet light or potassium permanganate solution. Radial

chromatography was carried out on 1mm, 2mm or 4mm plates of Merck Silica Gel 60 PF<sub>254</sub> containing gypsum, and visualised with short-wavelength ultraviolet light. Flash column chromatography was carried out under a positive pressure of nitrogen using Merck silica gel 60 (230 to 400 mesh). All reagents were purified by established techniques.<sup>1</sup> Petroleum ether and dichloromethane were distilled over calcium hydride, and ethyl acetate was distilled from phosphorous pentoxide before use. Petroleum ether here refers to the fraction collected between 60 and 80 °C. Diethyl ether and tetrahydrofuran were used freshly distilled from sodium benzophenone ketal, under a nitrogen atmosphere. Pyridine and triethylamine were distilled over calcium hydride, and pyridine was then stored over 4 Å molecular sieves. Dimethylformamide was dried by placing over activated 4 Å molecular sieves and standing for 24 h. This process was repeated four times before the dry DMF was stored over 4 Å molecular sieves under a nitrogen atmosphere. All other solvents were used as received from the supplier. Menadione, vitamin K2 and coenzyme Q-0 were obtained from Sigma Chemical Co. Geranyl bromide was obtained from Aldrich Chemical Co. Butyllithium was used fresh or titrated before use with *sec*-butanol and 1,10 phenanthroline to determine exact concentration.<sup>2</sup> All other reagents were obtained from commercial sources.

### 6.1.2 GENERAL METHODS

#### General Method A: Cuprate Coupling<sup>3</sup>

A solution of the aryl bromide (typically 1.8-2.5 mmol) in ether (to give a 0.6 M solution) was cooled to 0 °C under an argon atmosphere, and *n*BuLi (1.15 equiv) was added slowly. During this addition the reaction went green then dark red and after stirring at 0 °C for 30 min a pale precipitate formed. To this solution copper bromide dimethylsulfide complex (0.7 equiv) was added, and stirring continued for 2.5 h during which time the solution turned dark purple. To this the alkyl bromide (1.1 equiv) in ether (to give a 1 M solution) was slowly added and the reaction stirred at 0 °C for 2 h and room temperature for 3 h. The reaction was quenched via the addition of 10% aqueous HCl and the ether layer separated. The aqueous phase was extracted with ether (x3), and the combined organic fractions were washed with saturated

aqueous  $\text{NaHCO}_3$ , water, dried ( $\text{MgSO}_4$ ) and solvent removed to give the crude product.

#### **General Method B: Bromohydrin Formation<sup>4</sup>**

A solution of alkene (typically 0.1 to 1.6 mmol) in THF (to give a 0.03 M solution) was cooled in an ice bath and water was added slowly to the reaction until it remained turbid. THF was then added dropwise until the reaction just cleared. *N*-Bromosuccinimide (1.15 equiv) was added and the reaction stirred at room temperature for 3 h. The reaction was then extracted with ether (x3), dried ( $\text{MgSO}_4$ ) and solvent removed to give the crude product.

#### **General Method C: Epoxidation of Bromohydrin<sup>4</sup>**

To a stirred solution of bromohydrin (typically 0.05 to 0.9 mmol) in methanol (to give a 0.01 M solution) was added potassium carbonate (70 equiv) and the reaction was stirred at room temperature for 16 h. The reaction was filtered and solvent removed. Water was added and the resulting solution was extracted with dichloromethane, dried ( $\text{MgSO}_4$ ) and solvent removed under reduced pressure.

#### **General Method D: Epoxide Cleavage with Periodic Acid<sup>4</sup>**

To a stirred solution of epoxide (typically 0.05 to 0.9 mmol) in ether (to give a 0.02 M solution), was added a solution of periodic acid (1 equiv) in THF (to give a 0.04 M solution). This was stirred at room temperature for 1.5 h during which time a fine precipitate formed. The reaction was quenched via the addition of water, the ether layer was separated and the aqueous phase extracted with ether (x2). Combined organic fractions were dried ( $\text{MgSO}_4$ ) and solvent removed under reduced pressure.

#### **General Method E: Reduction of Aldehyde with Sodium Borohydride**

To a stirred solution of sodium borohydride (1 equiv) in methanol (to give a 0.5 M solution) at 0 °C, was added a solution of aldehyde (typically 0.05 to 0.3 mmol) in methanol (to give a 0.25 M solution). The reaction was stirred and allowed to warm to room temperature. After 20 min saturated aqueous  $\text{NH}_4\text{Cl}$  was added to quench the reaction, and methanol was removed under reduced pressure. The resulting aqueous phase was extracted with dichloromethane (x2), and the combined organic fractions

washed with saturated aqueous NaCl, water, dried (MgSO<sub>4</sub>) and solvent removed under reduced pressure.

#### **General Method F: Esterification with Succinic Anhydride<sup>5</sup>**

A solution of succinic anhydride (2.1 equiv), dimethylaminopyridine (0.05 equiv) and triethylamine (1.5 equiv) in dichloromethane (to give a 0.6 M solution), was added slowly to a solution of alcohol (typically 0.03 to 0.20 mmol) in dichloromethane (to give a 0.1 M solution). This was stirred at room temperature under an Ar atmosphere for 16 h. The reaction was diluted with dichloromethane and washed with 2N aqueous HCl, water, saturated aqueous NaHCO<sub>3</sub>, water, dried (MgSO<sub>4</sub>) and the solvent removed under reduced pressure.

#### **General Method G: Oxidative Deprotection with CAN<sup>3</sup>**

To a solution of analogue (typically 0.04 to 0.7 mmol) in a 2:1 acetonitrile:water mixture (to give a 0.25 M solution) was added a cooled solution of ceric ammonium nitrate (CAN) (2.5 equiv) in a 1:1 acetonitrile:water mixture (to give a 0.5 M solution), over a 10 min period. The resulting solution was stirred at 0 °C for 20 min and room temperature for 10 min before quenching via the addition of water. The resulting reaction mixture was extracted with dichloromethane (x2) and the combined organic fractions washed with water, dried (MgSO<sub>4</sub>) and the solvent removed under reduced pressure.

#### **General Method H: Peptide Coupling using EDCI<sup>6</sup>**

To a solution of substrate (typically 0.02 to 0.04 mmol) and peptide (1.1 equiv) in dichloromethane (typically 0.3 mL) stirring under an Ar atmosphere, was added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide. HCl (EDCI) (1.3 equiv), 1-hydroxybenzotriazole hydrate (1.5 equiv) and diisopropylethylamine (1.1 equiv). The resulting solution was stirred at room temperature for 16 h. The reaction was quenched via the addition of 3M aqueous NaCl solution and the layers separated. The aqueous phase was extracted with dichloromethane (x2), and combined organic fractions were dried (MgSO<sub>4</sub>), and the solvent removed under reduced pressure.

## 6.2 EXPERIMENTAL WORK DESCRIBED IN CHAPTER 2

The high-performance liquid chromatography work described in this chapter was carried out using a system made up of the following components; Shimadzu SCL-10A system controller, Shimadzu SIL-10AXL auto injector, Shimadzu LC-10AT liquid chromatograph pump, Shimadzu DGU-14A membrane degasser and Shimadzu RF-551 spectrofluorimetric detector. Unless otherwise stated the fluorescence detector was set at 'low' sensitivity. The arrangement of these components for each section of work is described below. The HPLC system was interfaced through Delta 5.0 Chromatography for data collection. All solvents used were HPLC grade, and used as received from the supplier. Where stated the analytical column used in this study was a 15 cm reverse-phase Merck Supersphere 100 RP-18 endcapped column.

### Investigation of Metal Catalysts

All metal catalysts were used as received from the supplier. A standard 0.5  $\mu\text{M}$  solution of vitamin K<sub>1</sub> in ethanol was used throughout this study. Before testing each catalyst was packed into an Alltech refillable guard column (20 mm x 2 mm) and flushed thoroughly with methanol at a flow rate of 0.5 mL/min for at least 1 h. A 400  $\mu\text{L}$  sample of vitamin K standard was injected into a degassed mobile phase of 100% MeOH and passed directly through the test catalytic column at a flow rate of 0.1 mL/min. This then passed through the fluorescence detector (excitation wavelength 249 nm and emission wavelength 408 nm), and the chromatogram was recorded. The total run time for this experiment was 15 min.

### Efficiency of Catalytic Reduction Column

A 0.5  $\mu\text{M}$  ethanolic solution of vitamin K<sub>1</sub> was used for this study. The mobile phase was 100% methanol, with an injection volume of 20  $\mu\text{L}$  and flow rate of 0.5 mL/min. The run time for these experiments was 25 minutes. The reference column used in this study was a platinum black catalytic column which consistently showed high activity, and which when placed in series with a second catalytic column showed no increase in peak area for a standard vitamin K injection. Two methods were used in this investigation.

## METHOD 1

The HPLC system was set up with the analytical RP column placed between the test catalytic column and the reference column. The vitamin K solution therefore, passed through the test column before separation of the quinone and hydroquinone on the RP column. This then passed through a second reference catalytic column and into the fluorescence detector. The recorded chromatogram showed two peaks at retention time 6.0 min for the hydroquinone and 21.3 min for the quinone. The area of the hydroquinone peak divided by the total area of the two peaks gave a measure of the reduction efficiency of the test column.

## METHOD 2

Alternatively, two HPLC runs were used for each test column. Initially the HPLC system was set up with the test column following the analytical RP column, and no catalytic column before the analytical RP column, and the peak area for vitamin K<sub>1</sub> was recorded. This process was then repeated replacing the test column with the reference column, and the peak area was again recorded. The ratio of the peak areas from these two chromatograms gave a measure of the reduction efficiency of the test column.

## Generality of Reduction System for Various Quinones

All quinones used were commercially available, and were used without purification. A 0.5  $\mu$ M solution of each quinone was prepared in HPLC grade ethanol. For this investigation the mobile phase was 100% methanol and a flow rate of 0.1 mL/min was used throughout. The HPLC system was set up to consist of the degasser, pump, autosampler, platinum black catalytic reduction column and fluorescence detector. The run time for this experiment was 15 min. A 400  $\mu$ L sample of each quinone was injected into the HPLC system. The fluorescence detector was set to run a time programme scanning over a range of excitation or emission wavelengths.

The following time programme used was:

Zero	0.05 minutes	- to zero the detector
Mark	3.99 minutes	- to mark the scan start
Scan	4.00 minutes	- scan over set wavelengths
End	15.00 minutes	- end run



Typical scanning wavelengths were 220-340 nm for excitation scans, and 350-470 nm for emission scans. As the scan speed, start time, and wavelength range were known for each experiment, the wavelength of  $\lambda_{\text{max}}$  for each scan could be calculated from the chromatogram obtained. As the fluorescence detector stored a scan after each experiment, the results obtained by calculation from the Delta chromatogram were compared with those obtained from the fluorescence detector in each case.

Once the excitation and emission maxima were obtained each quinone was rerun without the time programme, to obtain a measure of optimum fluorescence. As a control experiment each quinone was also tested in the absence of the platinum black catalyst to check for levels of native fluorescence of the oxidized form.

### **Effect of Solvent on Reduction System**

All experiments in this study were run using a 0.5  $\mu\text{M}$  ethanolic solution of vitamin K<sub>1</sub>. The RP column was fitted to the HPLC system described above for this study. The injection volume was 20  $\mu\text{L}$ , with a flow rate of 0.5 mL/min and excitation and emission wavelengths set at 249 nm and 408 nm respectively. Each solvent system was prepared and the HPLC thoroughly washed for 15 minutes and allowed to equilibrate. The peak area and retention time for vitamin K<sub>1</sub> were then recorded for each mobile phase investigated.

### **Linearity of Fluorescence**

A series of four concentrations of vitamin K<sub>1</sub> were prepared by dilution from the stock solution of vitamin K<sub>1</sub>. The final concentrations used in this study were 0.25, 0.5, 0.75 and 1.0  $\mu\text{M}$ . These experiments were run using the same HPLC set-up described above, with a mobile phase of 100% methanol and, excitation and emission wavelengths of 249 nm and 408 nm respectively. The run time for each injection was 25 minutes. Each standard solution was run in duplicate, and the experiment was repeated four times, using freshly prepared vitamin K<sub>1</sub> standards in each case. The peak areas from each chromatogram were recorded and used in the preparation of a calibration curve for vitamin K<sub>1</sub> through the given concentration range.

### Application to Biological Samples

For the detection of vitamin K in plasma samples, the sensitivity of the fluorescence detector was increased to 'high'. The injection volume was also increased from 20  $\mu\text{L}$  to 50  $\mu\text{L}$ . The HPLC set-up was the same as described above with a platinum black catalytic column used for the reduction of vitamin K, and a solvent system of 100% methanol. The sample extraction procedure used was a standard method used for other fat-soluble vitamins. This consisted of taking 400  $\mu\text{L}$  of plasma and adding 400  $\mu\text{L}$  of ethanol to precipitate the proteins. To this 400  $\mu\text{L}$  of hexane was added and the sample was vortexed to extract the vitamin K into the organic phase. The sample was centrifuged cold at 3000 rpm for 10 min, and 350  $\mu\text{L}$  of the hexane phase was removed and dried down under a stream of nitrogen. The resulting solid was reconstituted into 200  $\mu\text{L}$  of methanol for injection into the HPLC.

The standard solution of MK-4 used was prepared in ethanol from commercially available vitamin K<sub>2</sub>, at a concentration of 11 nM. A 50  $\mu\text{L}$  sample of this was injected into the HPLC as described above, and the resulting retention time and peak area recorded.

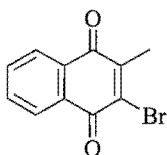
### Vitamin K Recoveries

A series of spiked plasma samples were prepared by diluting a stock solution of vitamin K<sub>1</sub> in ethanol to the required concentrations with pooled plasma. The final concentrations of these solutions were 5 nM, 10 nM, 20 nM and 50 nM. A sample of the unspiked plasma was also retained as a control. The preparation of these plasma samples before analysis by HPLC was carried out as describe above.

Analysis was carried out using the RP HPLC column, platinum black reactor column and fluorescence detection with Ex 249 and Em 408. The injection volume was 100  $\mu\text{L}$ , with the fluorescence detector set at 'high' sensitivity and a flow rate of 0.5 mL/min. Chromatograms were recorded, and results calculated, using Delta chromatography.

### 6.3 EXPERIMENTAL WORK DESCRIBED IN CHAPTER 3

#### 2-Bromo-3-methyl-1,4-naphthaquinone (3.19)<sup>7</sup>



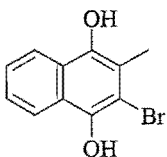
2-Methylnaphthaquinone (5.50g, 0.032 mol, 1 equiv) and anhydrous sodium acetate (11.00 g, 0.14 mol, 4.2 equiv) were dissolved in glacial acetic acid (50 mL) with heating to give a clear brown solution. This was cooled in ice and bromine (2.0 mL, 0.04 mol, 1.2 equiv) was added dropwise. The flask was then sealed, encased in foil and stored in the dark for 3 d, during which time yellow crystals formed. The entire contents of the flask was transferred to 300 mL of water and filtered. The resulting crude product was recrystallised from hot methanol to afford **3.19** as yellow needle-like crystals (5.69g, 71%); mp 149–150 °C (lit. mp 151-152 °C)<sup>7</sup>

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 2.40 (s, 3H, CH<sub>3</sub>), 7.75 (m, 2H, ArH), 8.15 (m, 2H, ArH)

<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 17.82, 127.06, 127.45, 131.14, 131.50, 133.85, 134.06, 139.00, 148.44, 177.45, 181.89

HRMS Found 249.9629 (Calcd for C<sub>11</sub>H<sub>7</sub>O<sub>2</sub><sup>79</sup>Br 249.9633)

#### 2-Bromo-3-methyl-1,4-naphthaquinol (3.61)<sup>7</sup>



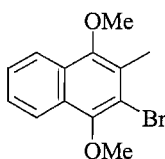
2-Bromo-3-methyl-1,4-naphthaquinone (**3.19**) (4.00g, 0.02 mol, 1 equiv) was dissolved in ethanol (55 mL) with heating. After cooling in ice to give a paste, was added a solution of stannous chloride (14.38g, 0.06 mol, 4 equiv) in conc. HCl (14.5 mL) was added. To this, water (45 mL) was added causing precipitation of the desired quinol, and the appearance of a strong purple colour. Upon heating the quinol redissolved and the solution cleared. Cooling gave **3.61** as pale pink crystals (3.347g). Concentration of the mother liquor and subsequent cooling gave a second

crop of pink crystals (386 mg, overall yield 93%); mp 101-102 °C (decomp.)

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  2.50 (s, 3H,  $\text{CH}_3$ ), 4.81 (s, 1H,  $\text{OH-1}$ ), 5.75 (s, 1H,  $\text{OH-4}$ ), 7.54 (m, 2H,  $\text{ArH}$ ), 8.04 (d,  $J=8.0$  Hz, 1H,  $\text{ArH}$ ), 8.20 (d,  $J=8.2$  Hz, 1H,  $\text{ArH}$ )

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  16.28, 107.96, 120.92, 122.44, 122.80, 125.70, 126.43, 127.09, 127.48, 133.87, 134.09

### 2-Bromo-3-methyl-1,4-dimethoxynaphthalene (**3.11**)<sup>7</sup>



A solution of dihydroxynaphthalene (**3.61**) (3.73g, 0.02 mol, 1 equiv) in dimethylsulfate (20 mL) was stirred vigorously under a nitrogen atmosphere. To this a solution of potassium hydroxide (33.1g, 0.59 mol, 40 equiv) in water (66 mL) was added portionwise with a dark colour appearing upon each addition. The addition was kept at a rate that avoided reflux of the reaction mixture. Upon completion of this addition the solution had turned orange and a precipitate had formed. The reaction was heated at 80 °C for 1 h and then allowed to cool slowly to room temperature, before dilution to 600 mL with water. The resulting solution was extracted with ether (3x) and the combined organic fraction washed with 5% aqueous NaOH, water, dried ( $\text{MgSO}_4$ ) and solvent removed to give orange crystals. Recrystallisation from hot methanol gave **3.11** as pale orange crystals (2.68g, 64%); mp 83-84 °C (lit. mp 84-85 °C)<sup>7</sup>

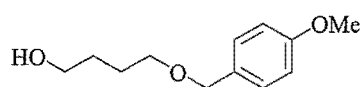
$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  2.54 (s, 3H,  $\text{CH}_3$ ), 3.88 (s, 3H,  $\text{OCH}_3$ ), 3.98 (s, 3H,  $\text{OCH}_3$ ), 7.52 (m, 2H,  $\text{ArH}$ ), 8.06 (m, 2H,  $\text{ArH}$ )

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  16.61, 61.22, 61.59, 117.19, 122.32, 122.36, 126.15, 126.48, 127.23, 127.51, 127.78, 149.80, 150.44

HREIMS Found 280.0095 (Calcd. for  $\text{C}_{13}\text{H}_{13}\text{O}_2^{79}\text{Br}$  280.0010)

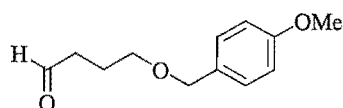
FTIR 3415.7, 2935.5, 1575.7  $\text{cm}^{-1}$

Anal. Calcd for  $\text{C}_{13}\text{H}_{13}\text{O}_2\text{Br}$ : C 55.5; H 4.7. Found: C 56.1; H 4.6.

**4-(4-Methoxybenzyloxy)butan-1-ol (3.63)<sup>8</sup>**

Sodium hydride (7.09 g of a 50% dispersion in oil, 0.09 mmol, 1.1 equiv) was prepared by washing four times with petroleum ether under an atmosphere of nitrogen. The sodium hydride was then dissolved in dry DMF (35 mL) and added to a solution of butan-1,4-diol (5 mL, 0.08 mol) in dry DMF (35 mL), stirring under nitrogen at  $-15\text{ }^{\circ}\text{C}$ . After 30 min, freshly prepared *p*-methoxybenzylbromide<sup>9</sup> (18.00 g, 0.09 mol, 1.15 equiv) in DMF (20 mL) was added. This was stirred at  $-15\text{ }^{\circ}\text{C}$  for a further 45 min, followed by stirring at room temperature for 1 h. The resulting solution was poured into an ice-water solution (200 mL) and extracted with ether (3 x 130 mL) during which time the milky white solution cleared. The organic fractions were combined and washed with saturated aqueous NaCl, dried ( $\text{MgSO}_4$ ) and the solvent removed to give crude product. This was purified by column chromatography eluting with a 1:1 mixture of ethyl acetate:petroleum ether. The desired protected alcohol (3.63) was isolated as a yellow oil (7.98 g, 48%).

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.68 (m, 4H,  $\text{HOCH}_2\text{CH}_2\text{CH}_2$ ), 3.49 (t,  $J=5.8$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 3.62 (t,  $J=5.9$  Hz, 2H,  $\text{HOCH}_2$ ), 3.79 (s, 3H,  $\text{OCH}_3$ ), 4.44 (s, 2H,  $\text{OCH}_2\text{Ar}$ ), 6.95 (d,  $J=8.8$  Hz, 2H,  $\text{ArH}$ ), 7.26 (d,  $J=8.8$  Hz, 2H,  $\text{ArH}$ )

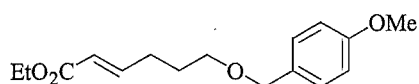
**4-(4-Methoxybenzyloxy)butanal (3.64)<sup>8</sup>**

A solution of dimethylsulphoxide (1 mL, 14.30 mmol, 3 equiv) in dichloromethane (12 mL) was stirred and cooled to  $-78\text{ }^{\circ}\text{C}$  under a nitrogen atmosphere. To this a solution of freshly distilled oxalyl chloride (0.84 mL, 9.50 mmol, 2 equiv) in dichloromethane (6 mL) was added slowly. This was stirred for 5 min before the addition of 3.63 (1 g, 4.76 mmol) in dichloromethane (8 mL). This was stirred at  $-78\text{ }^{\circ}\text{C}$  for 30 min before the dropwise addition of triethylamine (2.65 mL, 19.01 mmol, 4 equiv). The solution was warmed to room temperature and stirred for 1.5 h during

which time the milky white solution turned slightly yellow. The reaction was quenched via the addition of 5% aqueous HCl (24 mL) and the organic fraction collected and washed with saturated aqueous NaHCO<sub>3</sub>, saturated aqueous NaCl, and dried (MgSO<sub>4</sub>). Removal of the solvent under reduced pressure afforded **3.64** as a yellow oil (820 mg, 83%).

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.93 (m, 2H, CH<sub>2</sub>CH<sub>2</sub>O), 2.54 (t, *J*= 8.8 Hz, 2H, CHOCH<sub>2</sub>), 3.47 (t, *J*=6.3 Hz, 2H, CH<sub>2</sub>O), 3.80 (s, 3H, OCH<sub>3</sub>), 4.41 (s, 2H, CH<sub>2</sub>Ar), 6.87 (d, *J*=8.3 Hz, 2H, ArH), 7.23 (d, *J*=8.3 Hz, 2H, ArH), 9.77 (s, 1H, CHO)

**Ethyl 6-(4-methoxybenzyloxy)hex-2-enoate (3.65)**<sup>10</sup>

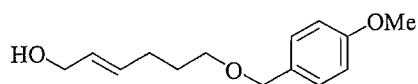


A solution of aldehyde (**3.64**) (50 mg, 2.40 mmol) and [(ethylcarbonyl)methylene]-triphenylphosphorane (16.4 mg, 0.47 mmol, 2 equiv) in dichloromethane (2 mL) was stirred under a nitrogen atmosphere. After 2 h the solvent was evaporated to give the crude product as an orange oil. This was purified by column chromatography eluting with 1:4 ethyl acetate:petroleum ether. The desired allylic ester (**3.65**) was isolated as an oil (54 mg, 81%).

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.27 (t, *J*=7.3 Hz, 3H, OCH<sub>2</sub>CH<sub>3</sub>), 1.74 (m, 2H, CH<sub>2</sub>CH<sub>2</sub>O), 2.28 (m, 2H, =CHCH<sub>2</sub>), 3.44 (t, *J*=6.0 Hz, 2H, CH<sub>2</sub>O), 3.77 (s, 3H, OCH<sub>3</sub>), 4.17 (q, *J*=7.3 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 4.41 (s, 2H, CH<sub>2</sub>Ar), 5.81 (d, *J*=15.5 Hz, 1H, COCH=), 6.86 (d, *J*=8.5 Hz, 2H, ArH), 6.89 (m, 1H, =CHCH<sub>2</sub>), 7.24 (d, *J*=8.5 Hz, 2H, ArH)

HREIMS Found 278.1512 (Calcd. for C<sub>16</sub>H<sub>22</sub>O<sub>4</sub> 278.1519)

**6-(4-Methoxybenzyloxy)hex-2-en-1-ol (3.66)**<sup>11</sup>



A solution of **3.65** (109 mg, 0.39 mmol) in dichloromethane (2 mL) was stirred under a nitrogen atmosphere at -78 °C. To this was added diisobutylaluminium hydride

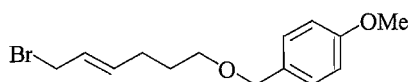
(123 mg, 0.86 mmol, 2.2 equiv) and the solution was stirred at  $-78\text{ }^{\circ}\text{C}$  for 30 min before warming to room temperature and stirring for 12 h. The reaction solution was slowly poured into a mixture of 2:1 ice:glacial acetic acid (15 mL) and stirred for 45 min until the solution cleared. The aqueous layer was separated and extracted with dichloromethane (x2) and the combined organic fractions were washed with saturated aqueous NaCl (x2), water, and dried ( $\text{MgSO}_4$ ). Solvent was removed under reduced pressure to afford **3.66** as a yellow oil (71 mg, 77%).

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.69 (m, 2H,  $=\text{CHCH}_2\text{CH}_2$ ), 2.12 (m, 2H,  $=\text{CHCH}_2$ ), 3.45 (t,  $J=6.3$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 3.80 (s, 3H,  $\text{OCH}_3$ ), 4.06 (d,  $J=4.9$  Hz, 2H,  $\text{HOCH}_2$ ), 4.43 (s, 2H,  $\text{OCH}_2\text{Ar}$ ), 5.65 (m, 2H,  $\text{CH}=\text{CH}$ ), 6.88 (dd,  $J=2.0$  and 6.8 Hz, 2H, ArH), 7.25 (dd,  $J=2.0$  and 6.8 Hz, 2H, ArH)

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  28.52, 28.80, 54.94, 62.95, 69.03, 72.19, 113.50, 129.06, 130.22, 131.88, 168.87

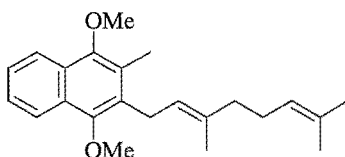
HREIMS found 236.1415 (Calcd. for  $\text{C}_{14}\text{H}_{20}\text{O}_3$  236.1412)

### 1-Bromo-6-(4-methoxybenzyloxy)hex-2-ene (**3.60**)<sup>11</sup>



To a stirred solution of **3.66** (1.302 g, 5.51 mmol) and carbon tetrabromide (2.28 g, 6.87 mmol, 1.25 equiv) in dichloromethane was added portionwise triphenylphosphine (2.17 g, 8.27 mmol, 1.5 equiv). During this addition the solution went pale orange and a fine precipitate appeared. This was stirred at room temperature for 5 min and the solvent removed under reduced pressure. This crude product was purified by column chromatography eluting with 1:4 ethyl acetate:petroleum ether, to afford **3.60** as a pale yellow oil (1.27 g, 77% yield).

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.69 (m, 2H,  $\text{CH}_2\text{CH}_2\text{O}$ ), 2.17 (m, 2H,  $=\text{CHCH}_2$ ), 3.44 (t,  $J=6.3$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 3.81 (s, 3H,  $\text{OCH}_3$ ), 3.93 (d,  $J=6.3$  Hz, 2H,  $\text{BrCH}_2$ ), 4.43 (s, 2H,  $\text{OCH}_2\text{Ar}$ ), 5.73 (m, 2H,  $\text{CH}=\text{CH}$ ), 6.88 (dd,  $J=2.0$  and 6.8 Hz, 2H, ArH), 7.26 (dd,  $J=2.0$  and 6.8 Hz, 2H, ArH)

**2-(3,7-Dimethylocta-2,6-dienyl)-3-methyl-1,4-dimethoxynaphthalene (3.67)<sup>3</sup>**


General method A was carried out using bromonaphthalene **3.11** (500 mg, 1.78 mmol, 1 equiv) and *n*BuLi (1.36 mL of 1.6 M solution in hexane, 2.00 mmol, 1.15 equiv). To this was added copper bromide dimethylsulfide complex (256 mg, 1.24 mmol, 0.7 equiv) and geranyl bromide (0.39 mL, 1.96 mmol, 1.1 equiv). Work up afforded the crude product as an orange oil which was purified by radial chromatography eluting with 1:20 ethyl acetate:petroleum ether. This gave an orange oil (509 mg) containing a mixture of **3.67** and 2-methyl-1,4-dimethoxynaphthalene (10:1 by <sup>1</sup>H NMR).

<sup>1</sup>H NMR (CDCl<sub>3</sub>) for 2-methyl-1,4-dimethoxynaphthalene: δ 2.45 (s, 3H, ArCH<sub>3</sub>), 3.86 (s, 3H, OCH<sub>3</sub>), 3.97 (s, 3H, OCH<sub>3</sub>), 6.61 (s, 1H, ArH), 7.46 (m, 2H, ArH), 8.01 (d, *J* = 8.3 Hz, 1H, ArH), 8.19 (d, *J* = 8.3 Hz, 1H, ArH)

<sup>1</sup>H NMR (CDCl<sub>3</sub>) for **3.67**: δ 1.56 (s, 3H, CH<sub>3</sub>), 1.63 (s, 3H, CH<sub>3</sub>), 1.82 (s, 3H, =CCH<sub>3</sub>), 2.03 (m, 4H, 2x CH<sub>2</sub>), 2.37 (s, 3H, ArCH<sub>3</sub>), 3.56 (d, *J* = 5.4 Hz, 2H, ArCH<sub>2</sub>), 3.87 (s, 3H, OCH<sub>3</sub>), 3.88 (s, 3H, OCH<sub>3</sub>), 5.10 (m, 2H, CH=C), 7.45 (m, 2H, ArH), 8.03 (m, 2H, ArH)

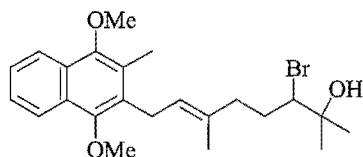
<sup>13</sup>C NMR (CDCl<sub>3</sub>) for **3.67**: δ 12.27, 16.26, 25.61, 26.27, 26.49, 34.17, 61.19, 62.08, 121.39, 122.04, 122.21, 122.90, 124.19, 125.20, 125.32, 126.37, 126.87, 127.20, 131.27, 135.56, 149.67, 150.01

HREIMS Found 338.2248 (Calcd. for C<sub>23</sub>H<sub>30</sub>O<sub>2</sub> 338.2246)

FTIR 2931.6, 1593.1 cm<sup>-1</sup>



**3-Bromo-8-(3-methyl-1,4-dimethoxynaphthalen-2-yl)-2,6-dimethylocta-6-en-2-ol (3.76)**



General method B was carried out with **3.67** (562 mg, 1.60 mmol, 1 equiv) and *N*-bromosuccinimide (340 mg, 1.90 mmol, 1.15 equiv) in THF (60mL). Work up and purification by radial chromatography eluting with 1:9 ethyl acetate:petroleum ether to 1:1 ethyl acetate:petroleum ether afforded **3.76** as a yellow oil (425 mg, 59%)

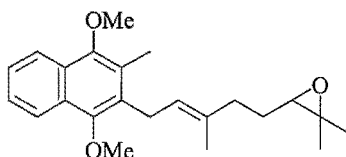
$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.31 (s, 3H,  $\text{CH}_3$ ), 1.33 (s, 3H,  $\text{CH}_3$ ), 1.81 (m, 1H,  $\text{CH}_{2a}\text{CBr}$ ), 1.84 (s, 3H,  $=\text{CCH}_3$ ), 2.02 (m, 1H,  $\text{CH}_{2b}\text{CBr}$ ), 2.15 (m, 1H,  $=\text{CCH}_{2a}$ ), 2.35 (m, 1H,  $=\text{CCH}_{2b}$ ), 2.40 (s, 3H,  $\text{ArCH}_3$ ), 3.59 (d,  $J=6.4$  Hz, 2H,  $\text{ArCH}_2$ ), 3.88 (s, 3H,  $\text{OCH}_3$ ), 3.90 (s, 3H,  $\text{OCH}_3$ ), 3.93 (dd,  $J=1.9$  and 11.2 Hz, 1H,  $\text{CHBr}$ ), 5.22 (t,  $J=6.4$  Hz, 1H,  $\text{CH=}$ ), 7.46 (m, 2H,  $\text{ArH}$ ), 8.06 (m, 2H,  $\text{ArH}$ )

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.46, 16.25, 25.93, 26.30, 26.4, 31.98, 38.15, 61.29, 62.15, 70.51, 72.40, 122.09, 122.21, 124.37, 125.29, 125.43, 126.69, 127.19, 127.49, 130.49, 133.90, 149.70, 150.12

HRFABMS Found 434.1463 (Calcd. For  $\text{C}_{23}\text{H}_{31}\text{O}_3^{79}\text{Br}$  434.1454)

FTIR 3465.8, 2933.5, 2841.0, 1739.7, 1591.2, 1456.2  $\text{cm}^{-1}$

**3-[5-(3-Methyl-1,4-dimethoxynaphthalen-2-yl)-3-methylpent-3-enyl]-2,2-dimethyloxirane (3.73)<sup>12</sup>**



General method C was carried out using bormohydrin (**3.76**) (380 mg, 0.87 mmol) and potassium carbonate (8.4 g, 0.06 mol, 70 equiv). Work up gave **3.73** as a pale yellow oil (280 mg, 91%), which was not purified further.

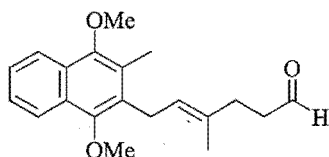
$^1\text{H}$  NMR ( $\text{CDCl}_3$ ) 1.23 (s, 3H,  $\text{CH}_3$ ), 1.24 (s, 3H,  $\text{CH}_3$ ), 1.65 (m, 2H,  $=\text{CCH}_2$ ), 1.85 (s, 3H,  $=\text{CCH}_3$ ), 2.13 (m, 2H,  $\text{CH}_2\text{CH}_2\text{CO}$ ), 2.37 (s, 3H,  $\text{ArCH}_3$ ), 2.67 (t,  $J=6.4$  Hz, 1H,  $\text{CHO}$ ), 3.57 (d,  $J=6.4$  Hz, 2H,  $\text{ArCH}_2$ ), 3.86 (s, 3H,  $\text{OCH}_3$ ), 3.88 (s, 3H,  $\text{OCH}_3$ ), 5.17 (t,  $J=1.5$  and  $6.4$  Hz, 2H,  $\text{CH}=\text{CH}$ ), 7.45 (m, 2H,  $\text{ArH}$ ), 8.04 (m, 2H,  $\text{ArH}$ )

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.41, 16.38, 18.68, 24.79, 26.31, 27.42, 36.30, 58.28, 61.29, 62.15, 64.07, 122.10, 122.24, 123.37, 125.28, 125.42, 126.73, 127.22, 127.49, 130.58, 134.82, 149.73, 150.10

HREIMS Found 354.2203 (Calcd. for  $\text{C}_{23}\text{H}_{30}\text{O}_3$  354.2195)

FTIR 2993.5, 2841.0, 1591.2, 1456.2  $\text{cm}^{-1}$

### 6-(3-Methyl-1,4-dimethoxynaphthalen-2-yl)-4-methylhex-4-enal (3.77)



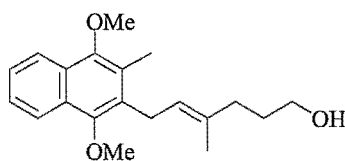
General method D was carried out using epoxide **3.73** (275 mg, 0.78 mmol) and periodic acid (177 mg, 0.78 mmol, 1 equiv) in THF (22 mL). Work up afforded **3.77** as yellow oil (217 mg, 90%), which was not purified further.

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.84 (s, 3H,  $=\text{CCH}_3$ ), 2.35 (s, 3H,  $\text{ArCH}_3$ ), 2.36 (m, 2H,  $=\text{CCH}_2$ ), 2.52 (t,  $J=2.0$  Hz, 2H,  $\text{CH}_2\text{CHO}$ ), 3.56 (d,  $J=6.4$  Hz, 2H,  $\text{ArCH}_2$ ), 3.86 (s, 3H,  $\text{OCH}_3$ ), 3.87 (s, 3H,  $\text{OCH}_3$ ), 5.15 (t,  $J=6.4$  Hz, 1H,  $=\text{CH}$ ), 7.46 (m, 2H,  $\text{ArH}$ ), 8.03 (m, 2H,  $\text{ArH}$ ), 9.72 (s, 1H,  $\text{CHO}$ )

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.39, 16.45, 26.24, 31.70, 42.05, 61.30, 62.15, 122.09, 122.21, 123.88, 125.32, 125.47, 126.66, 127.17, 127.51, 130.28, 133.69, 149.72, 150.10, 202.32

HREIMS Found 312.1725 (Calcd. for  $\text{C}_{20}\text{H}_{24}\text{O}_3$  312.1725)

FTIR 2933.5, 2839.0, 1591.2  $\text{cm}^{-1}$

**6-(3-Methyl-1,4-dimethoxynaphthalen-2-yl)-4-methylhex-4-en-1-ol (3.78)**

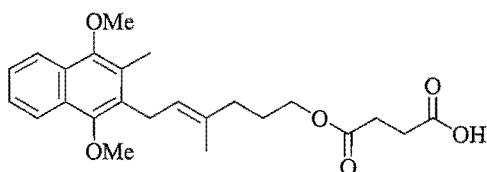
General method E was carried out using sodium borohydride (1.8 mg, 0.05 mmol) and **3.77** (15 mg, 0.05 mmol, 1 equiv). Work up afforded **3.78** as a yellow oil (9 mg, 60%), which was not purified further.

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.67 (m, 2H,  $\text{CH}_2\text{CH}_2\text{CH}_2$ ), 1.84 (s, 3H,  $\text{CCH}_3$ ), 2.07 (t,  $J=7.3$  Hz, 2H,  $=\text{CCH}_2$ ), 2.37 (s, 3H,  $\text{ArCH}_3$ ), 3.57 (t,  $J=5.4$  Hz, 2H,  $\text{CH}_2\text{OH}$ ), 3.59 (d,  $J=12.7$  Hz, 2H,  $\text{ArCH}_2$ ), 3.87 (s, 3H,  $\text{OCH}_3$ ), 3.88 (s, 3H,  $\text{OCH}_3$ ), 5.15 (dt,  $J=1.5$  and 6.4 Hz, 1H,  $\text{CH}=\text{)$ , 7.45 (m, 2H,  $\text{ArH}$ ), 8.05 (m, 2H,  $\text{ArH}$ )

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.42, 16.29, 26.30, 30.81, 35.88, 61.30, 62.15, 62.71, 122.09, 122.24, 123.19, 125.29, 125.42, 126.75, 127.22, 127.48, 130.65, 135.32, 149.73, 150.10

HREIMS Found 314.1883 (Calcd. For  $\text{C}_{20}\text{H}_{26}\text{O}_3$  314.1882)

FTIR 3346.3, 2937.4, 1591.2, 1456.2  $\text{cm}^{-1}$

**6-(3-Methyl-1,4-dimethoxynaphthalen-2-yl)-4-methylhex-4-enyl hydrogen succinate (3.79)**

General method F was carried out using succinic anhydride (68 mg, 0.68 mmol, 2.1 equiv), dimethylaminopyridine (2 mg, 0.02 mmol, 0.05 equiv), triethylamine (0.07 mL, 0.49 mmol, 1.5 equiv) and the alcohol **3.78** (80 mg, 0.32 mmol, 1 equiv). Work up afforded **3.79** as a yellow solid (76mg, 68%), which was not purified further. Mp 97-99  $^{\circ}\text{C}$

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.73 (m, 2H,  $\text{CH}_2\text{CH}_2\text{CH}_2$ ), 1.82 (s, 3H,  $=\text{CCH}_3$ ), 2.05 (t,  $J=2.4$  Hz,  $=\text{CCH}_2$ ), 2.36 (s, 3H,  $\text{ArCH}_3$ ), 2.59 (m, 4H, 2x  $\text{CH}_2$ ), 3.55 (d,  $J=6.4$  Hz, 2H,

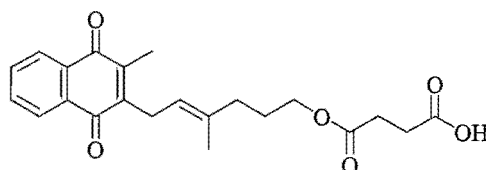
ArCH<sub>2</sub>), 3.87 (s, 3H, OCH<sub>3</sub>), 3.88 (s, 3H, OCH<sub>3</sub>), 4.05 (t, *J*= 6.8 Hz, 2H, CH<sub>2</sub>O), 5.13 (t, *J*=6.4 Hz, 1H, CH=), 7.45 (m, 2H, ArH), 8.04 (m, 2H, ArH)

<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 12.37, 16.22, 26.26, 26.88, 29.76, 30.93, 35.73, 61.23, 62.09, 64.64, 122.06, 122.21, 123.37, 125.25, 125.39, 126.70, 127.17, 127.45, 130.51, 134.53, 149.68, 150.06, 174.20, 180.61

HRFABMS Found 414.2043 (Calcd. for C<sub>24</sub>H<sub>30</sub>O<sub>6</sub> 414.2044)

FTIR 2937.4, 1733.9, 1591.2 cm<sup>-1</sup>

**6-(3-Methyl-1,4-naphthoquinon-2-yl)-4-methylhex-4-enyl hydrogen succinate (3.70)**

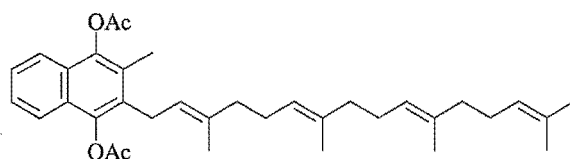


General method G was carried out using **3.79** (12 mg, 0.04 mmol) and ceric ammonium nitrate (53 mg, 0.10 mmol, 2.5 equiv). Work up and purification by radial chromatography eluting with dichloromethane to 1:19 MeOH:dichloromethane afforded **3.70** as yellow oil (8 mg, 72%).

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.70 (m, 2H, CH<sub>2</sub>CH<sub>2</sub>O), 1.78 (s, 3H, =CCH<sub>3</sub>), 2.03 (t, *J*=7.8 Hz, 2H, =CCH<sub>2</sub>), 2.18 (s, 3H, ArCH<sub>3</sub>), 2.58 (m, 2H, CH<sub>2</sub>CO<sub>2</sub>H), 2.63 (m, 2H, COCH<sub>2</sub>), 3.35 (d, *J*=6.8 Hz, 2H, ArCH<sub>2</sub>), 4.03 (t, *J*=6.4 Hz, 2H, CH<sub>2</sub>O), 5.04 (dt, *J*=1.5 and 6.8 Hz, 1H, CH=), 7.68 (m, 2H, ArH), 8.06 (m, 2H, ArH)

<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 12.69, 16.22, 25.98, 26.64, 28.80 (x2), 35.70, 64.35, 119.84, 126.16, 126.27, 132.04 (x2), 133.32, 133.35, 136.26, 143.43, 145.82, 172.12, 177.62, 184.52, 185.40

HRFABMS Found 384.1565 (Calcd. for C<sub>22</sub>H<sub>24</sub>O<sub>6</sub> 384.1572)

**16-(3-Methyl-1,4-diacetoxynaphthalen-2-yl)-2,6,10,14-tetramethylhexadeca-2,6,10,14-tetraene (3.85)**

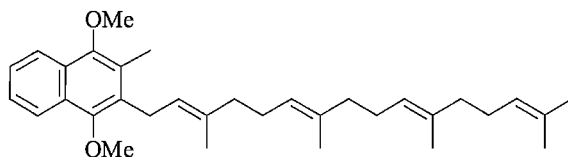
To a refluxing solution of vitamin K<sub>2</sub> (250 mg, 0.56 mmol, 1 equiv) in acetic anhydride (2.5 mL) was added zinc powder slowly so a slight excess of solid zinc was always present. The reaction mixture was refluxed for 45 mins before the addition of sodium acetate (160 mg, 1.95 mmol, 3.5 equiv). After refluxing for a further 15 mins the reaction was poured into ice and extracted with ethyl acetate. The combined organic fractions were washed with water, saturated aqueous NaHCO<sub>3</sub>, water, dried (MgSO<sub>4</sub>) and solvent removed to give **3.85** as a yellow oil (295 mg, 99%), which was not further purified.

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.57 (s, 6H, =C(CH<sub>3</sub>)<sub>2</sub>), 1.59 (s, 3H, CH<sub>3</sub>), 1.67 (s, 3H, CH<sub>3</sub>), 1.78 (s, 3H, CH<sub>3</sub>), 1.95-2.09 (m, 12H, 6x CH<sub>2</sub>), 2.22 (s, 3H, ArCH<sub>3</sub>), 2.46 (s, 3H, COCH<sub>3</sub>), 2.48 (s, 3H, COCH<sub>3</sub>), 3.41 (d, *J*=4.4 Hz, 2H, ArCH<sub>2</sub>), 5.06-5.09 (m, 4H, 4x CH=), 7.46 (m, 2H, ArH), 7.68 (m, 2H, ArH)

<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 13.04, 16.00, 16.34, 17.64, 20.59, 20.66, 22.14, 25.66, 26.54, 26.62, 26.74, 27.02, 39.58, 39.66, 39.69, 121.09, 121.16, 121.37, 123.92, 124.22, 124.37, 126.21, 126.30, 128.39, 128.72, 130.31, 131.20, 133.58, 133.83, 134.85, 135.14, 136.27, 142.26, 166.37, 168.97

HREIMS Found 530.3395 (Calcd. For C<sub>35</sub>H<sub>46</sub>O<sub>4</sub> 530.3396)

**16-(3-Methyl-1,4-dimethoxynaphthalen-2-yl)-2,6,10,14-tetramethylhexadeca-2,6,10,14-tetraene (3.89)<sup>13</sup>**



**METHOD A**

To a solution of vitamin K<sub>2</sub> (50 mg, 0.11 mmol) and tetrabutylammoniumiodide (4.3 mg, 0.01 mmol, 0.12 equiv) in tetrahydrofuran (0.3 mL) and water (0.1 mL) was added a solution of sodium dithionite (117 mg, 0.67 mmol, 6 equiv) in water (0.3 mL). This was stirred at room temperature under a nitrogen atmosphere for 15 min before the slow addition of potassium hydroxide (145 mg, 2.60 mmol, 23 equiv) in water (0.16 mL). The reaction was stirred for 5 min during which time the solution turned orange, dark red and dark green. Dimethylsulfate (0.13 mL, 1.30 mmol, 12 equiv) was added and the solution went orange. This was stirred at room temperature for 18 h. The reaction mixture was extracted with dichloromethane (x2) and combined organic fractions washed with water dried (MgSO<sub>4</sub>) and solvent removed to give crude product. Purification by radial chromatography eluting with 1:19 ethyl acetate:pet ether afforded **3.89** as a yellow oil (30 mg, 56%).

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.59 (s, 3H, CH<sub>3</sub>), 1.60 (s, 3H, CH<sub>3</sub>), 1.61 (s, 3H, CH<sub>3</sub>), 1.69 (s, 3H, CH<sub>3</sub>), 1.86 (s, 3H, CH<sub>3</sub>), 1.95-2.12 (m, 12H, 6x CH<sub>2</sub>), 2.40 (s, 3H, ArCH<sub>3</sub>), 3.59 (d, *J*=6.3 Hz, 2H, ArCH<sub>2</sub>), 3.89 (s, 3H, OCH<sub>3</sub>), 3.91 (s, 3H, OCH<sub>3</sub>), 5.09-5.14 (m, 4H, CH=), 7.47 (m, 2H, ArH), 8.07 (m, 2H, ArH)

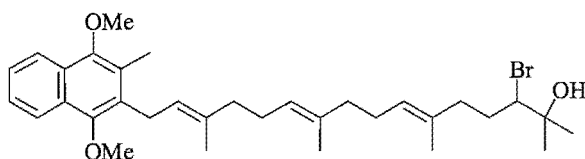
<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 12.35, 15.91, 15.97, 16.36, 17.63, 25.64, 26.30, 26.52, 26.59, 26.72, 30.84, 39.66, 61.25, 62.12, 122.06, 122.22, 122.80, 124.02, 124.16, 124.37, 125.21, 125.34, 126.87, 127.22, 127.44, 130.85, 131.16, 134.84, 135.03, 135.68, 149.70, 150.05

HREIMS Found 474.3492 (Calcd. for C<sub>33</sub>H<sub>46</sub>O<sub>2</sub> 474.3498)

## METHOD B

A solution of vitamin K<sub>2</sub> (250 mg, 0.56 mmol) in ether (10 mL) was added via cannular to a solution of sodium dithionite (3.92 g, 0.02 mol, 40 equiv) in water (39 mL). The resulting solution was stirred vigorously under a nitrogen atmosphere for 30 min before the layers were allowed to separate and the ether layer was transferred via cannular to a nitrogen flushed two-necked flask. The original solution was washed with ether and the combined organic layers evaporated to give a dark red solid. This was suspended in dimethylsulfate (0.75 mL, 7.90 mmol, 14 equiv) and stirred under nitrogen. A solution of potassium hydroxide (1.26 g, 0.02 mol, 40 equiv) in water (2.90 mL) was added dropwise with stirring. Once addition was complete the reaction was heated at 80 °C for 1 h and allowed to cool slowly. The reaction was diluted with water and extracted (3x) with diethyl ether. The combined organic fractions were washed with water, 10% NaOH, water, dried (MgSO<sub>4</sub>) and solvent removed to give a crude product which was purified by radial chromatography eluting with 1:19 ethyl acetate:petroleum ether. The desired product **3.89** was isolated as a yellow oil (89 mg, 33%). Data for this compound is given above.

**3-Bromo-16-(3-methyl-1,4-dimethoxynaphthalen-2-yl)-2,6,10,14-tetramethyl-hexadeca-6,4,10-trien-2-ol (3.90)**



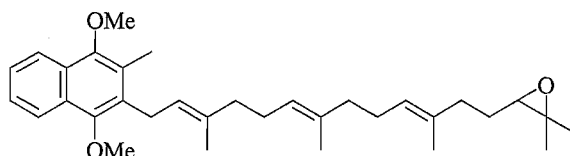
General method B was carried out using **3.89** (35 mg, 0.07 mmol) and *N*-bromosuccinimide (15 mg, 0.08 mmol, 1.15 equiv). Work up and purification by radial chromatography eluting with 1:9 ethyl acetate:petroleum ether gave **3.90** as a yellow oil (21 mg, 50%)

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.32 (s, 3H, CH<sub>3</sub>), 1.33 (s, 3H, CH<sub>3</sub>), 1.56 (s, 3H, CH<sub>3</sub>), 1.58 (s, 3H, CH<sub>3</sub>), 1.84 (s, 3H, CH<sub>3</sub>), 1.93-2.10 (m, 12H, 6x CH<sub>2</sub>), 2.38 (s, 3H, ArCH<sub>3</sub>), 3.57 (d, *J*=6.3 Hz, 2H, ArCH<sub>2</sub>), 3.87 (s, 3H, OCH<sub>3</sub>), 3.89 (s, 3H, OCH<sub>3</sub>), 3.95 (dd, *J*=2.0 and 11.2 Hz, 1H, CHBr), 5.09-5.16 (m, 3H, CH=), 7.45 (m, 2H, ArH), 8.06 (m, 2H, ArH)

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.35, 15.75, 15.94, 16.36, 25.79, 26.29, 26.52, 32.07, 38.10, 39.54, 39.63, 61.25, 62.12, 70.78, 72.40, 122.06, 122.22, 122.82, 124.20, 125.22, 125.35, 125.86, 126.87, 127.20, 127.42, 130.85, 132.94, 134.80, 135.65, 149.68, 150.04

HREIMS Found 490.3457 (-HBr) (Calcd for  $\text{C}_{33}\text{H}_{46}\text{O}_3$  490.3449)

**3-[13-(3-Methy-1,4-dimethoxynaphthalen-2-yl)-3,7,11-trimethyltrideca-3,7,11-trienyl]-2,2-dimethyloxirane (3.91)**



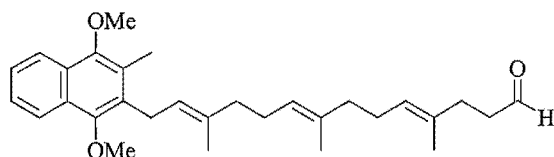
General method C was carried out using **3.90** (32 mg, 0.06 mmol) and potassium carbonate (542 mg, 3.92 mmol, 70 equiv). Work up afforded **3.91** as a yellow oil (22 mg, 80%), which was not purified further.

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.25 (s, 3H,  $\text{CH}_3$ ), 1.29 (s, 3H,  $\text{CH}_3$ ), 1.57 (s, 3H,  $\text{CH}_3$ ), 1.58 (s, 3H,  $\text{CH}_3$ ), 1.83 (s, 3H,  $\text{CH}_3$ ), 1.94-2.10 (m, 12H, 6x  $\text{CH}_2$ ), 2.38 (s, 3H,  $\text{ArCH}_3$ ), 2.69 (t,  $J=6.4$  Hz, 1H,  $\text{CH}_2\text{O}$ ), 3.57 (d,  $J=6.4$  Hz, 2H,  $\text{ArCH}_2$ ), 3.87 (s, 3H,  $\text{OCH}_3$ ), 3.89 (s, 3H,  $\text{OCH}_3$ ), 5.10 (m, 3H,  $\text{CH=}$ ), 7.45 (m, 2H,  $\text{ArH}$ ), 8.06 (m, 2H,  $\text{ArH}$ )

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.35, 15.91, 15.96, 16.36, 18.68, 24.83, 26.29, 26.51, 26.56, 27.42, 36.23, 39.56, 39.63, 58.26, 61.25, 62.12, 64.14, 122.06, 122.22, 122.80, 124.10, 124.78, 125.21, 125.34, 126.86, 127.20, 127.42, 130.84, 133.94, 134.88, 135.64, 149.69, 150.04

HREIMS Found 490.3456 (Calcd. for  $\text{C}_{33}\text{H}_{46}\text{O}_3$  490.3449)



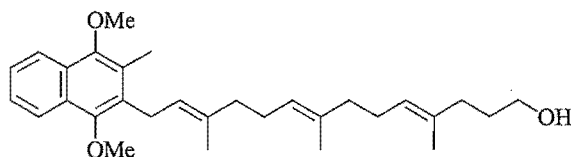
**14-(3-Methyl-1,4-dimethoxynaphthalen-2-yl)-4,8,12-trimethyltetradeca-4,8,12-trienal (3.92)**

General method D was carried out using **3.91** (22 mg, 0.04 mmol) and periodic acid (10 mg, 0.04 mmol, 1 equiv). Work up afforded **3.92** as a yellow oil (19 mg, 95%), which was not purified further.

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.56 (s, 3H,  $\text{CH}_3$ ), 1.57 (s, 3H,  $\text{CH}_3$ ), 1.83 (s, 3H,  $\text{CH}_3$ ), 1.95-2.07 (m, 8H, 4x  $\text{CH}_2$ ), 2.27 (t,  $J=7.3$  Hz, 2H,  $\text{CH}_2\text{CH}_2\text{CHO}$ ), 2.38 (s, 3H,  $\text{ArCH}_3$ ), 2.47 (t,  $J=7.3$  Hz, 2H,  $\text{CH}_2\text{CHO}$ ), 3.57 (d,  $J=6.3$  Hz, 2H,  $\text{ArCH}_2$ ), 3.87 (s, 3H,  $\text{OCH}_3$ ), 3.88 (s, 3H,  $\text{OCH}_3$ ), 5.09 (m, 3H,  $\text{CH=}$ ), 7.45 (m, 2H,  $\text{ArH}$ ), 8.05 (m, 2H,  $\text{ArH}$ ), 9.71 (s, 1H,  $\text{CHO}$ )

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.35, 15.93, 15.98, 16.36, 26.29, 26.47, 31.78, 39.43, 39.62, 42.08, 61.26, 62.13, 67.91, 122.06, 122.22, 122.82, 124.24, 125.22, 125.29, 125.35, 126.87, 127.20, 127.44, 130.84, 132.82, 134.71, 135.62, 149.69, 150.04, 202.67

HREIMS Found 448.2980 (Calcd. for  $\text{C}_{30}\text{H}_{40}\text{O}_3$  448.2978)

**14-(3-Methyl-1,4-dimethoxynaphthalen-2-yl)-4,8,12-trimethyltetradeca-4,8,12-trien-1-ol (3.93)**

General method E was carried out using **3.92** (31 mg, 0.07 mmol) and sodium borohydride (4 mg, 0.07 mmol, 1 equiv). The reaction was followed by TLC and after 20 min at room temperature starting material was still present, so more sodium borohydride (4 mg, 1 equiv) was added and the reaction stirred for a further 20 min. Work up afforded **3.93** as a yellow oil (23 mg, 74%), which was not further purified.

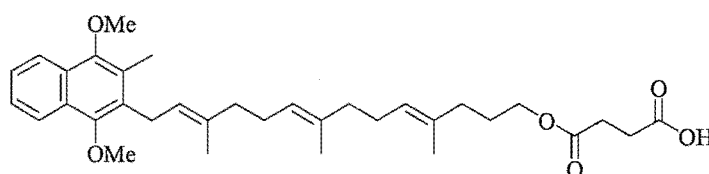
$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.56 (s, 3H,  $\text{CH}_3$ ), 1.57 (s, 3H,  $\text{CH}_3$ ), 1.64 (m, 2H,  $\text{CH}_2\text{CH}_2\text{OH}$ ),

1.83 (s, 3H, CH<sub>3</sub>), 1.92-2.28 (m, 10H, 5x CH<sub>2</sub>), 2.38 (s, 3H, ArCH<sub>3</sub>), 3.58 (t, *J*=5.9 Hz, 2H, CH<sub>2</sub>OH), 3.61 (d, *J*=6.3 Hz, 2H, ArCH<sub>2</sub>), 3.87 (s, 3H, OCH<sub>3</sub>), 3.89 (s, 3H, OCH<sub>3</sub>), 5.05-5.13 (m, 3H, 3x CH=), 7.45 (m, 2H, ArH), 8.05 (m, 2H, ArH)

<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 12.35, 15.76, 15.93, 16.36, 26.30, 26.45, 29.66, 30.67, 35.91, 39.56, 39.62, 61.26, 62.13, 62.71, 122.06, 122.22, 122.82, 124.13, 124.66, 125.22, 125.35, 126.88, 127.20, 127.44, 130.85, 134.53, 134.88, 135.65, 149.68, 150.04

HREIMS Found 450.3143 (Calcd for C<sub>30</sub>H<sub>42</sub>O<sub>3</sub> 450.3134)

**14-(3-Methyl-1,4-dimethoxynaphthalen-2-yl)-4,8,12-trimethyltetradeca-4,8,12-trienyl hydrogen succinate (3.94)**



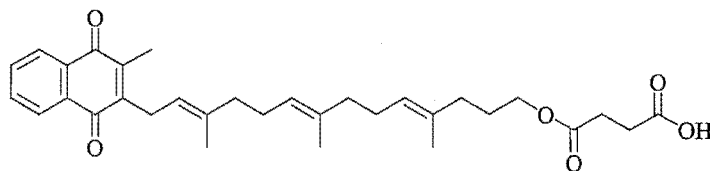
General method F was carried out using succinic anhydride (5 mg, 0.05 mmol, 2.1 equiv), dimethylaminopyridine (0.15 mg, 0.05 equiv), triethylamine (5 μL, 0.04 mmol, 1.5 equiv) and the alcohol **3.93** (11 mg, 0.02 mmol, 1 equiv). Work up and purification by radial chromatography eluting with 1:4 ethyl acetate:petroleum ether afforded **3.94** as yellow oil (8 mg, 60%).

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.56 (s, 6H, 2x CH<sub>3</sub>), 1.69 (m, 2H, CH<sub>2</sub>CH<sub>2</sub>O), 1.82 (s, 3H, CH<sub>3</sub>), 1.92-2.10 (m, 10H, 5x CH<sub>2</sub>), 2.37 (s, 3H, ArCH<sub>3</sub>), 2.65 (m, 4H, C=OCH<sub>2</sub>CH<sub>2</sub>C=O), 3.56 (d, *J*=6.3 Hz, 2H, ArCH<sub>2</sub>), 3.87 (s, 3H, OCH<sub>3</sub>), 3.89 (s, 3H, OCH<sub>3</sub>), 4.05 (t, *J*=6.8 Hz, 2H, CH<sub>2</sub>O), 5.06 (m, 3H, CH=), 7.45 (m, 2H, ArH), 8.06 (m, 2H, ArH)

<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 12.35, 15.78, 15.97, 16.37, 26.30, 26.52, 26.59, 26.77, 29.22, 35.66, 39.58, 39.62, 50.74, 51.84, 61.29, 62.15, 64.57, 122.06, 122.22, 122.76, 124.09, 124.98, 125.25, 125.38, 126.90, 127.20, 127.44, 130.88, 133.62, 134.95, 135.72, 149.68, 173.55

HRMS Found 550.3311 (Calcd. for C<sub>34</sub>H<sub>46</sub>O<sub>6</sub> 550.3294)

**14-(3-Methyl-1,4-naphthoquinon-2-yl)-4,8,12-trimethyltetradeca-4,8,12-trienyl hydrogen succinate (3.80)**

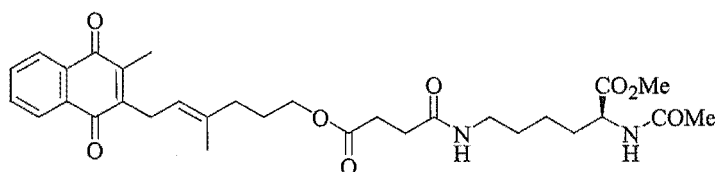


General method G was carried out using ceric ammonium nitrate (17 mg, 0.03 mmol, 2.5 equiv) and **3.94** (7 mg, 0.01 mmol, 1 equiv). Work up and purification by radial chromatography eluting with dichloromethane afforded **3.80** as a yellow oil (4 mg, 60%).

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.55 (s, 6H, 2x  $\text{CH}_3$ ), 1.69 (m, 2H,  $\text{CH}_2\text{CH}_2\text{O}$ ), 1.79 (s, 3H  $\text{CH}_3$ ), 1.94-2.05 (m, 10H, 5x  $\text{CH}_2$ ), 2.19 (s, 3H,  $\text{ArCH}_3$ ), 2.65 (m, 4H,  $\text{COCH}_2\text{CH}_2\text{CO}$ ), 3.37 (d,  $J=6.8$  Hz, 2H,  $\text{ArCH}_2$ ), 4.05 (t,  $J=6.0$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 5.04 (m, 3H, 3x  $\text{CH=}$ ), 7.68 (m, 2H,  $\text{ArH}$ ), 8.08 (m, 2H,  $\text{ArH}$ )

## 6.4 EXPERIMENTAL WORK DESCRIBED IN CHAPTER 4

**Methyl 2-acetyl-amino-6-[4-methyl-6-(3-methyl-1,4-naphthoquinon-2-yl)hex-4-enoylamino]hexanoate (4.2)**



General method H was carried out using analogue **3.70** (10 mg, 0.03 mmol), and *N*- $\alpha$ -acetyl-lysine methyl ester hydrochloride (7 mg, 0.03 mmol, 1.1 equiv). Extraction and purification by radial chromatography eluting with 1:19 MeOH:dichloromethane afforded **4.2** (7 mg, 55%).

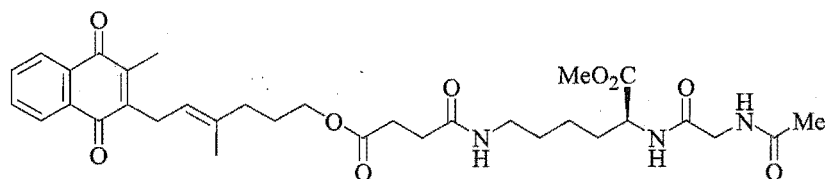
$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.35 (m, 2H,  $\text{NHCH}_2\text{CH}_2$ ), 1.51 (m, 2H,  $\text{NHCH}_2\text{CH}_2\text{CH}_2$ ), 1.69 (m, 1H,  $\text{CH}_{2a}\text{CHCO}_2\text{Me}$ ), 1.71 (m, 2H,  $\text{CH}_2\text{CH}_2\text{O}$ ), 1.80 (s, 3H,  $=\text{CCH}_3$ ), 1.82 (m, 1H,  $\text{CH}_{2b}\text{CHCO}_2\text{Me}$ ), 2.04 (s, 3H,  $\text{COCH}_3$ ), 2.05 (t,  $J=14.2$  Hz, 2H,  $=\text{CCH}_2$ ), 2.19 (s,

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  2.43 (t,  $J=6.8$  Hz, 2H,  $\text{CH}_2\text{CONH}$ ), 2.64 (dt,  $J=5.4$  and  $6.8$  Hz, 2H,  $\text{OCOCH}_2$ ), 3.20 (m, 1H,  $\text{NHCH}_{2a}$ ), 3.26 (m, 1H,  $\text{NHCH}_{2b}$ ), 3.37 (d,  $J=7.3$  Hz, 2H,  $\text{ArCH}_2$ ), 3.74 (s, 3H,  $\text{OCH}_3$ ), 4.02 (t,  $J=6.3$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 4.56 (m, 1H,  $\text{CHCO}_2\text{Me}$ ), 5.04 (dt,  $J=1.0$  and  $6.8$  Hz, 1H,  $\text{CH=}$ ), 5.94 (brs, 1H,  $\text{NH}$ ), 6.37 (brs, 1H,  $\text{NH}$ ), 7.70 (m, 2H,  $\text{ArH}$ ), 8.08 (m, 2H,  $\text{ArH}$ )

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.71, 16.26, 22.11, 23.06, 26.00, 26.67, 28.77, 29.66, 30.95, 31.75, 35.74, 38.74, 51.83, 52.38, 64.31, 119.84, 126.19, 126.28, 132.09, 133.34, 133.38, 136.31, 143.45, 145.85, 170.31, 171.77, 172.94, 173.06, 184.53, 185.43

HRESMS ( $\text{M}+\text{H}$ ) Found 591.2687 (Calcd. for  $\text{C}_{31}\text{H}_{41}\text{N}_2\text{O}_8$  569.2863)

**Methyl 2-(2-acetylamino-acetylamino)-6-[4-methyl-6-(3-methyl-1,4-naphthoquinon-2-yl)hex-4-enoylamino]hexanoate (4.4)**



General method H was carried out using analogue (3.70) (15 mg, 0.04 mmol) and *N*-acetyl-gly-lys methyl ester acetate (14 mg, 0.04 mmol, 1.1 equiv). Extraction and purification by radial chromatography eluting with 1:19 MeOH:dichloromethane afforded 4.4 as an orange oil (12 mg, 50%).

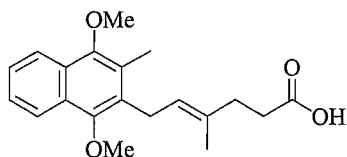
$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.34 (m, 2H,  $\text{NHCH}_2\text{CH}_2$ ), 1.47 (m, 2H,  $\text{NHCH}_2\text{CH}_2\text{CH}_2$ ), 1.70 (t,  $J=6.8$  Hz,  $\text{CH}_2\text{CH}_2\text{O}$ ), 1.70-1.85 (m, 2H,  $\text{CH}_2\text{CHCO}_2\text{Me}$ ), 1.78 (s, 3H,  $=\text{CCH}_3$ ), 2.03 (s, 3H,  $\text{COCH}_3$ ), 2.04 (t,  $J=9.8$  Hz, 2H,  $=\text{CCH}_2$ ), 2.18 (s, 3H,  $\text{ArCH}_3$ ), 2.44 (m, 2H,  $\text{COCH}_2$ ), 2.62 (m, 2H,  $\text{CH}_2\text{CO}$ ), 3.15 (m, 1H,  $\text{NHCH}_{2a}$ ), 3.26 (m, 1H,  $\text{NHCH}_{2b}$ ), 3.35 (d,  $J=6.8$  Hz,  $\text{ArCH}_2$ ), 3.72 (s, 3H,  $\text{OCH}_3$ ), 3.88 (dd,  $J=4.9$  and  $16.6$  Hz, 1H,  $\text{COCH}_{2a}\text{NH}$ ), 4.01 (t,  $J=6.8$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 4.04 (dd,  $J=4.9$  and  $16.6$  Hz, 1H,  $\text{COCH}_{2b}\text{NH}$ ), 4.54 (m, 1H,  $\text{CHCO}_2\text{Me}$ ), 5.02 (dt,  $J=1.5$  and  $6.8$  Hz, 1H,  $\text{CH=}$ ), 6.25 (m, 1H,  $\text{NH}$ ), 6.74 (m, 1H,  $\text{NH}$ ), 7.04 (m, 1H,  $\text{NH}$ ), 7.69 (m, 2H,  $\text{ArH}$ ), 8.06 (m, 2H,  $\text{ArH}$ )

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.72, 16.27, 21.97, 22.89, 25.99, 26.66, 28.54, 29.48, 30.74, 31.24, 35.73, 38.57, 43.11, 51.91, 52.45, 64.36, 119.81, 126.20, 126.28, 132.07,

133.36, 133.40, 136.32, 143.47, 145.85, 169.14, 170.98, 171.86, 172.51, 173.33, 184.56, 185.44

HRESMS (M+H) found 626.3079 (Calcd. For  $C_{33}H_{44}N_3O_9$  626.3078)

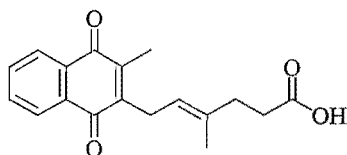
**6-(3-Methyl-1,4-dimethoxynaphthalen-2-yl)-4-methylhex-4-enoic acid (4.8)<sup>14</sup>**



A solution of **3.77** (120 mg, 0.38 mmol) in ethanol (2.4 mL) was added to a solution of silver nitrate (89 mg, 0.58 mmol, 1.5 equiv) in ethanol (4.2 mL) and stirred under a nitrogen atmosphere. To this a solution of sodium hydroxide (0.2 mL of 5N aqueous solution) in ethanol (2.4 mL) was added dropwise over a ten min period. The resulting black solution was stirred overnight at room temperature. The reaction mixture was filtered and the ethanol removed. Water was added and the solution was extracted with ethyl acetate (x3). The combined organic fractions were dried ( $MgSO_4$ ) and solvent removed to give **4.8** as a yellow oil (62 mg, 50%), which was not purified further.

$^1H$  NMR ( $CDCl_3$ )  $\delta$  1.85 (s, 3H,  $=CCH_3$ ), 2.33 (m, 2H,  $=CCH_2$ ), 2.35 (s, 3H,  $ArCH_3$ ), 2.44 (m, 2H,  $CH_2CO_2H$ ), 3.56 (d,  $J=6.4$  Hz, 2H,  $ArCH_2$ ), 3.86 (s, 3H,  $OCH_3$ ), 3.87 (s, 3H,  $OCH_2$ ), 5.17 (t,  $J=6.4$  Hz, 1H,  $=CH$ ), 7.46 (m, 2H,  $ArH$ ), 8.04 (m, 2H,  $ArH$ )

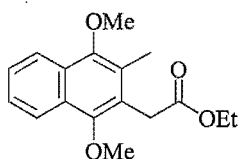
$^{13}C$  NMR ( $CDCl_3$ )  $\delta$  12.34, 16.26, 26.23, 32.74, 34.20, 60.45, 61.27, 62.15, 122.0, 122.20, 123.92, 125.28, 125.44, 126.72, 127.46, 130.36, 133.67, 177.34, 179.41, 213.29

**6-(3-Methyl-1,4-naphthoquinon-2-yl)-4-methylhex-4-enoic acid (4.5)**<sup>14</sup>

General method G was carried out using CAN (84 mg, 0.15 mmol, 2.5 equiv) and **4.8** (20 mg, 0.06 mmol). Work up and purification by radial chromatography eluting with dichloromethane afforded **4.5** as a yellow oil (11 mg, 60%).

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.81 (s, 3H, =CCH<sub>3</sub>), 2.17 (s, 3H, ArCH<sub>3</sub>), 2.29 (m, 2H, =CCH<sub>2</sub>), 2.41 (m, 2H, CH<sub>2</sub>CO<sub>2</sub>H), 3.36 (d, *J*=6.8 Hz, 2H, ArCH<sub>2</sub>), 5.06 (t, *J*=6.4 Hz, 1H, CH=), 7.68 (m, 2H, ArH), 8.06 (m, 2H, ArH)

<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 12.70, 16.28, 25.93, 32.55, 34.27, 120.32, 126.19, 126.32, 132.07, 133.34, 133.38, 135.45, 143.57, 145.67, 178.57, 184.45, 185.36

**Ethyl 2-(3-methyl-1,4-dimethoxynaphthalen-2-yl)acetate (4.9)**

General method A was carried out using **3.11** (500 mg, 1.78 mmol), nBuLi (1.26 mL of 1.6 M solution in hexane, 2.00 mmol, 1.15 equiv), copper bromide dimethylsulfide complex (256 mg, 1.24 mmol, 0.7 equiv) and ethyl bromoacetate (0.22 mL, 1.96 mmol 1.1 equiv). Standard work up gave a crude product which, upon by radial chromatography eluting with 1:19 ethyl acetate:petroleum ether to 1:4 ethyl acetate:petroleum ether, afforded **4.9** as a yellow oil (174 mg, 34%).

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.26 (t, *J*=5.4 Hz, 3H, OCH<sub>2</sub>CH<sub>3</sub>), 2.36 (s, 3H, ArCH<sub>3</sub>), 3.87 (s, 3H, OCH<sub>3</sub>), 3.91 (s, 3H, OCH<sub>3</sub>), 3.92 (s, 2H, ArCH<sub>2</sub>), 4.19 (q, *J*=7.3 Hz, 2H, OCH<sub>2</sub>CH<sub>3</sub>), 7.48 (m, 2H, ArH), 8.06 (m, 2H, ArH)

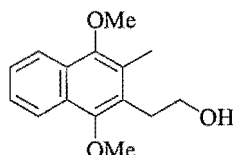
<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 12.54, 14.13, 32.97, 60.80, 61.30, 62.23, 122.13, 122.36, 124.01, 125.37, 125.92, 126.54, 126.93, 128.17, 150.01, 150.66, 171.64

HREIMS Found 288.1366 (Calcd. for  $C_{17}H_{20}O_4$  288.1362)

Anal. Calcd for  $C_{17}H_{20}O_4$ : C 70.81; H 6.99. Found C 70.51; H 7.03.

FTIR 2987.5, 2941.2, 2837.1, 1732.0, 1595.0  $cm^{-1}$

**2-(3-Methyl-1,4-dimethoxynaphthalen-2-yl)ethanol (4.10)**



To a solution of ester (4.9) (110 mg, 0.38 mmol) in diethyl ether (3.60 mL), stirred under an argon atmosphere, was added lithium aluminiumhydride (29 mg, 0.76 mmol, 2 equiv) and the reaction was stirred at room temperature for 20 min. TLC analysis showed the reaction to be incomplete so additional lithium aluminiumhydride (10 mg) was added and the reaction stirred for a further 30 min. After this time no starting material was present by TLC. The reaction was quenched via the addition of saturated aqueous  $NH_4Cl$  and the organic layer separated. The aqueous phase was extracted with ethyl acetate and the combined organic fractions washed with water, brine, dried ( $MgSO_4$ ) and solvent removed. Purification by radial chromatography eluting with 1:4 ethyl acetate:petroleum ether to 1:1 ethyl acetate:petroleum ether gave 4.10 as a white solid (85 mg, 90%), which was used without further purification. Mp 66–69 °C.  $R_f$  (4.9) = 0.61,  $R_f$  (4.10) = 0.14, 1:4 ethyl acetate:petroleum ether.

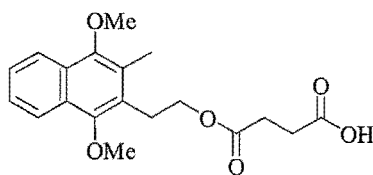
$^1H$  NMR ( $CDCl_3$ )  $\delta$  2.43 (s, 3H,  $ArCH_3$ ), 3.12 (t,  $J=6.8$  Hz, 2H,  $ArCH_2$ ), 3.85 (t,  $J=6.8$  Hz, 2H,  $CH_2OH$ ), 3.86 (s, 3H,  $OCH_3$ ), 3.92 (s, 3H,  $OCH_3$ ), 7.47 (m, 2H  $ArH$ ), 8.04 (m, 2H,  $ArH$ )

$^{13}C$  NMR ( $CDCl_3$ )  $\delta$  12.54, 30.61, 61.32, 61.87, 62.62, 122.17, 122.19, 125.46, 125.72, 126.43, 126.91, 127.33, 127.81, 150.32, 150.50

HREIMS Found 246.1255 (Calcd. For  $C_{15}H_{18}O_3$  246.1256)

Anal. Calcd for  $C_{15}H_{18}O_3$ : C 73.1; H 7.4. Found C 72.8; H 7.4.

FTIR 3265.3, 2935.5, 2841.0, 1589.2  $cm^{-1}$

**2-(3-Methyl-1,4-dimethoxynaphthalen-2-yl)ethyl hydrogen succinate (4.11)**

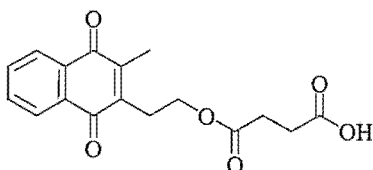
General method F was carried out using succinic anhydride (23 mg, 0.23 mmol, 2.1 equiv), dimethylaminopyridine (1 mg, 0.05 equiv), triethylamine (0.02 mL, 0.16 mmol, 1.5 equiv) and **4.10** (27 mg, 0.11 mmol). Work up afforded **4.11** as a yellow oil (21 mg, 55%), with no further purification.

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  2.45 (s, 3H,  $\text{ArCH}_3$ ), 2.66 (q,  $J=4.9$  Hz, 4H, 2x  $\text{CH}_2$ ), 3.17 (t,  $J=7.3$  Hz, 2H,  $\text{ArCH}_2$ ), 3.86 (s, 3H,  $\text{OCH}_3$ ), 3.91 (s, 3H,  $\text{OCH}_3$ ), 4.29 (t,  $J=7.3$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 5.29 (s, 1H, OH), 7.47 (m, 2H,  $\text{ArH}$ ), 8.03 (m, 2H,  $\text{ArH}$ )

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.48, 26.68, 28.85 (x2), 61.30, 62.14, 63.93, 122.18, 122.27, 125.43, 125.81, 126.12, 126.49, 127.01, 127.92, 150.15, 150.89, 172.13, 177.93

HRFABMS found 346.1420 (Calcd. For  $\text{C}_{19}\text{H}_{22}\text{O}_6$  346.1416)

Anal. Calcd for  $\text{C}_{19}\text{H}_{22}\text{O}_6$ : C 65.9; H 6.4. Found: C 66.3; H 6.8.

**2-(3-Methyl-1,4-naphthoquinon-2-yl)ethyl hydrogen succinate (4.7)**

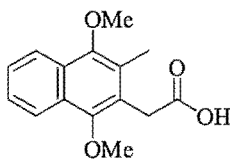
General method G was carried out using **4.11** (58 mg, 0.17 mmol) and ceric ammonium nitrate (230 mg, 0.42 mmol, 2.5 equiv). Work up and purification by radial chromatography eluting with dichloromethane to 1:19 MeOH:petroleum ether afforded **4.7** as a bright yellow oil (40 mg, 76%).

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  2.19 (s, 3H,  $\text{ArCH}_3$ ), 2.55 (m, 4H, 2x  $\text{CH}_2$ ), 2.96 (t,  $J=5.9$  Hz, 2H,  $\text{ArCH}_2$ ), 4.21 (t,  $J=6.4$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 7.66 (m, 2H,  $\text{ArH}$ ), 8.02 (m, 2H,  $\text{ArH}$ )

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  12.92, 26.60, 28.66 (x2), 62.88, 126.23 (x2), 131.80, 131.91, 133.44, 133.47, 142.49, 145.41, 172.06, 177.27, 184.29, 184.86

HRFABMS found 315.0874 (Calcd for  $\text{C}_{17}\text{H}_{15}\text{O}_6$  315.0869)



**2-(3-Methyl-1,4-dimethoxynaphthalen-2-yl)acetic acid (4.12)**<sup>15</sup>

A solution of **4.9** (58mg, 0.20 mmol) and potassium carbonate (47 mg, 0.34 mmol, 1.2 equiv) in 10:1 MeOH:water (5.5 mL) was refluxed for 18 h during which time the solution went orange. The MeOH was removed under reduced pressure and 10% aqueous HCl (5 mL) was added with vigorous stirring. The reaction mixture was extracted with ethyl acetate (x2), combined organic fractions were dried (MgSO<sub>4</sub>) and solvent removed to give **4.12** as an orange solid (38 mg, 73%).

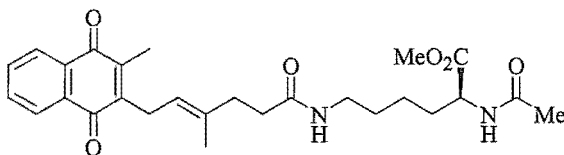
<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 2.40 (s, 3H, ArCH<sub>3</sub>), 3.88 (s, 3H, OCH<sub>3</sub>), 3.93 (s, 3H, OCH<sub>3</sub>), 3.98 (s, 2H, ArCH<sub>2</sub>), 7.50 (m, 2H, ArH), 8.07 (m, 2H, ArH), 9.89 (brs, 1H, OH)

<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 12.64, 32.71, 61.41, 62.31, 122.24, 122.40, 123.16, 125.57, 126.16, 126.42, 126.88, 128.37, 150.11, 150.75, 178.06

HREIMS Found 260.1047 (Calcd for C<sub>15</sub>H<sub>16</sub>O<sub>4</sub> 260.1049)

Anal. Calcd for C<sub>15</sub>H<sub>16</sub>O<sub>4</sub>; C 69.2; H 6.2. Found: C 69.0; H 6.3.

FTIR 3423.4, 2922.0, 1701.1 cm<sup>-1</sup>

**Methyl 2-acetylamino-6-[4-methyl-6-(3-methyl-1,4-naphthoquinon-2-yl)hex-4-enoylamino]hexanoate (4.13)**

General method H was carried out using analogue **4.5** (6 mg, 0.02 mmol) was reacted with *N*-α-acetyl-lysine methyl ester hydrochloride (5 mg, 0.02 mmol, 1.1 equiv), and after extraction and purification by radial chromatography eluting with 1:19 MeOH:dichloromethane afforded **4.13** as an orange oil (5 mg, 51%).

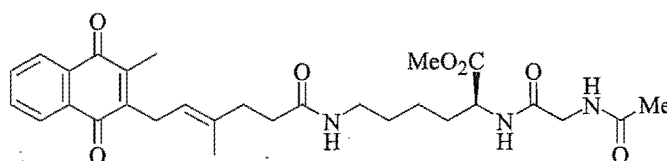
<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.28 (m, 2H, NHCH<sub>2</sub>CH<sub>2</sub>), 1.46 (m, 2H, NHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 1.68 (m, 1H, CH<sub>2a</sub>CHNH), 1.80 (m, 1H, CH<sub>2b</sub>CHNH), 1.81 (s, 3H, =CCH<sub>3</sub>), 2.02 (s, 3H,

COCH<sub>3</sub>), 2.17 (s, 3H, ArCH<sub>3</sub>), 2.28 (m, 4H, CH<sub>2</sub>CH<sub>2</sub>CO), 3.17 (m, 2H, NHCH<sub>2</sub>), 3.36 (d, *J*=6.8 Hz, 2H, ArCH<sub>2</sub>), 3.73 (s, 3H, OCH<sub>3</sub>), 4.53 (m, 1H, CHCO<sub>2</sub>Me), 5.06 (t, *J*=6.4 Hz, 1H, CH=), 5.72 (brs, 1H, NH), 6.32 (d, *J*=7.3 Hz, 1H, NH), 7.70 (m, 2H, ArH), 8.07 (m, 2H, ArH)

<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 12.73, 16.38, 22.21, 23.13, 26.07, 28.84, 31.85, 35.18, 35.29, 38.70, 51.81, 52.41, 120.16, 126.26, 126.69, 132.07 (x2), 133.43 (x2), 136.24, 143.56, 145.60, 170.20, 172.97, 172.99, 184.79, 185.35

HRESMS (M+H) found 483.2504 (Calcd. for C<sub>27</sub>H<sub>35</sub>N<sub>2</sub>O<sub>6</sub> 483.2495)

**Methyl 2-(2-acetylamino-acetylamino)-6-[4-methyl-6-(3-methyl-1,4-naphthoquinon-2-yl)hex-4-enoylamino]hexanoate (4.14)**



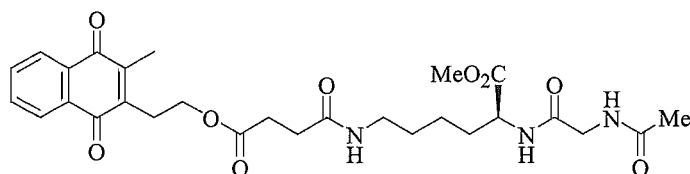
General method H was carried out using analogue **4.5** (9 mg, 0.03 mmol) and *N*-acetyl-gly-lys methyl ester acetate (11 mg, 0.03 mmol, 1.1 equiv). Following extraction and purification by radial chromatography eluting with 1:19 MeOH:dichloromethane **4.14** was isolated as an orange oil (5 mg, 31%).

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.24 (m, 2H, NHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 1.40 (m, 2H, NHCH<sub>2</sub>CH<sub>2</sub>), 1.65 (m, 1H, CH<sub>2a</sub>CHNH), 1.78 (s, 3H, =CCH<sub>3</sub>), 1.80 (m, 1H, CH<sub>2b</sub>CHNH), 2.03 (s, 3H, COCH<sub>3</sub>), 2.16 (s, 3H, ArCH<sub>3</sub>), 2.28 (m, 4H, =CCH<sub>2</sub>CH<sub>2</sub>), 3.12 (m, 2H, NHCH<sub>2</sub>), 3.33 (d, *J*=6.8 Hz, 2H, ArCH<sub>2</sub>), 3.70 (s, 3H, OCH<sub>3</sub>), 3.90 (dd, *J*=4.9 and 19.0 Hz, 1H, COCH<sub>2a</sub>NH), 4.00 (dd, *J*=4.9 and 19.0 Hz, 1H, COCH<sub>2b</sub>NH), 4.50 (m, 1H, CHCO<sub>2</sub>Me), 5.04 (t, *J*=5.9 Hz, 1H, CH=), 6.17 (brs, 1H, NH), 6.80 (brs, 1H, NH), 7.10 (d, *J*=7.3 Hz, 1H, NH), 7.68 (m, 2H, ArH), 8.06 (m, 2H, ArH)

<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 12.70, 16.23, 22.87, 26.04, 28.71, 29.66, 31.14, 35.04, 35.26, 38.68, 43.17, 52.06, 52.44, 120.18, 126.21, 126.25, 126.63, 132.04 (x2), 133.42 (x2), 136.11, 143.57, 145.67, 171.37, 172.40, 173.49, 184.58, 185.31

HRESMS (M+H) found 540.2706 (Calcd. for C<sub>29</sub>H<sub>38</sub>N<sub>3</sub>O<sub>7</sub> 540.2710)

**Methyl 2-(2-acetylamino-acetylamino)-6-{3-[2-(3-methyl-1,4-naphthoquinon-2-yl)ethoxycarbonyl]propionylamino}hexanoate (4.15)**



General method H was carried out using **4.7** (10 mg, 0.03 mmol) and *N*-acetyl-gly-lys methyl ester acetate (11 mg, 0.03 mmol, 1.1 equiv). Following extraction and purification by radial chromatography eluting with 1:19 MeOH:dichloromethane **4.15** was isolated as an orange oil (15 mg, 85%).

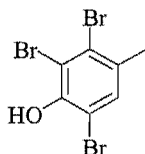
$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.34 (m, 2H,  $\text{NHCH}_2\text{CH}_2\text{CH}_2$ ), 1.47 (m, 2H,  $\text{NHCH}_2\text{CH}_2$ ), 1.69 (m, 1H,  $\text{CH}_{2a}\text{CHNH}$ ), 1.84 (m, 1H,  $\text{CH}_{2b}\text{CHNH}$ ), 2.03 (s, 3H,  $\text{COCH}_3$ ), 2.24 (s, 3H,  $\text{ArCH}_3$ ), 2.61 (m, 4H,  $\text{COCH}_2\text{CH}_2\text{CO}$ ), 3.01 (t,  $J=6.8$  Hz, 2H,  $\text{ArCH}_2\text{CH}_2$ ), 3.14 (m, 1H,  $\text{NHCH}_{2a}$ ), 3.24 (m, 1H,  $\text{NHCH}_{2b}$ ), 3.72 (s, 3H,  $\text{OCH}_3$ ), 3.90 (dd,  $J=5.4$  and 17.1 Hz, 1H,  $\text{COCH}_{2a}\text{NH}$ ), 4.03 (dd,  $J=5.4$  and 17.1 Hz, 1H,  $\text{COCH}_{2b}\text{NH}$ ), 4.27 (t,  $J=6.8$  Hz, 2H,  $\text{ArCH}_2$ ), 4.54 (m, 1H,  $\text{CHCO}_2\text{Me}$ ), 6.41 (m, 1H,  $\text{NH}$ ), 6.86 (m, 1H,  $\text{NH}$ ), 7.16 (d,  $J=7.8$  Hz, 1H,  $\text{NH}$ ), 7.71 (m, 2H,  $\text{ArH}$ ), 8.08 (m, 2H,  $\text{ArH}$ )

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  13.00, 22.06, 22.76, 26.63, 28.49, 29.38 (x2), 31.22, 38.65, 43.01, 51.91, 52.41, 62.88, 126.30, 126.34, 131.89, 132.05, 133.55 (x2), 142.55, 145.57, 169.25, 171.25, 171.77, 172.60, 173.16, 184.48, 184.95

HRESMS ( $\text{M}+\text{H}$ ) Found 558.2464 (Calcd. for  $\text{C}_{28}\text{H}_{36}\text{N}_3\text{O}_9$  558.2452)

## 6.5 EXPERIMENTAL WORK DESCRIBED IN CHAPTER 5

### 2,3,6-Tribromo-4-methylphenol (5.47)<sup>16</sup>

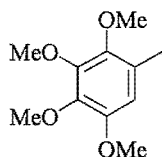


To a solution of *p*-cresol (25g, 0.23 mol) in chloroform (27 mL) containing iron filings (0.9 g, 0.02 mol, 0.07 equiv) was added bromine (37 ml, 0.72 mol, 3.1 equiv) dropwise, over 5 h at room temperature. During this addition a large amount of HBr gas was produced which was trapped by bubbling through water. The reaction solution turned dark red and solidified upon addition of the last 4-5 mL of bromine. More chloroform (5 mL) was added and the reaction stirred for 48 h. The reaction was filtered, the filtrate washed with dilute aqueous sodium hydrogen sulfite and dried (MgSO<sub>4</sub>). Solvent was removed under reduced pressure to afford a crude product which was recrystallised from petroleum ether to give **5.47** (48.54 g) as off-white needles, in 61% yield. Mp 96-98 °C (lit. mp 102 °C)<sup>16</sup>

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 2.39 (s, 3H, ArCH<sub>3</sub>), 5.90 (s, 1H, OH), 7.39 (s, 1H, ArH)

HREIMS Found 341.7901 (Calcd. for C<sub>7</sub>H<sub>5</sub>O<sup>79</sup>Br<sub>3</sub> 341.7891)

### 1-Methyl-2,3,4,5-tetramethoxybenzene (5.48)<sup>16</sup>



2,3-Dimethoxy-5-methyl-1,4-benzoquinone (1g, 5.49 mmol, 1 equiv) was dissolved in hot ethanol and cooled rapidly in ice. To this was added a solution of stannous chloride (4.95g, 0.02 mol, 4 equiv) in conc. HCl (5mL) during which time the red colour faded to pale yellow. This was extracted with ethyl acetate (3x), dried (MgSO<sub>4</sub>) and solvent removed to give pale yellow crystals. This was suspended in dimethylsulfate (7.2 mL, 0.08 mmol, 14 equiv) and stirred in a two-necked flask

under nitrogen. To this was added portionwise a solution of potassium hydroxide (12 g, 0.22 mol, 40 equiv) in water (26 mL) not allowing the solution to reflux. During this addition a thick white precipitate formed, which then redissolved leaving a yellow solution that faded to white. Upon completion of the addition of potassium hydroxide the reaction was heated to 80 °C under a nitrogen atmosphere for 1 h, then allowed to cool slowly. The reaction was diluted with water (180 mL) and extracted with ether (3x). The combined organic fractions were washed with 5% aqueous NaOH, water, dried (MgSO<sub>4</sub>) and solvent removed to afford **5.48** as a pale yellow oil (990 mg, 85%), which was not purified further.

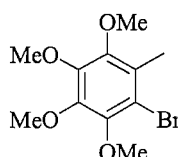
<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 2.22 (s, 3H, ArCH<sub>3</sub>), 3.78 (s, 3H OCH<sub>3</sub>), 3.81 (s, 3H OCH<sub>3</sub>), 3.86 (s, 3H OCH<sub>3</sub>), 3.93 (s, 3H OCH<sub>3</sub>), 6.44 (s, 1H, ArH)

<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 15.84, 56.10, 60.65, 61.08, 61.17, 108.31, 125.89, 140.79, 145.40, 147.00, 149.11

HREIMS Found 212.1057 (Calcd. For C<sub>11</sub>H<sub>16</sub>O<sub>4</sub> 212.1049)

FTIR 2937.4, 2837.1, 1591.2 cm<sup>-1</sup>

### 1-Bromo-2-methyl-3,4,5,6-tetramethoxybenzene (**5.42**)<sup>16</sup>



To a stirred solution of **5.48** (490 mg, 2.30 mmol, 1 equiv) in dichloromethane (2.3 mL) at 3-5 °C was added slowly a solution of bromine (0.12 mL, 2.30 mmol, 1 equiv) in dichloromethane (0.46 mL). The resulting dark orange solution was stirred for a further 5 min. The reaction mixture was washed with water (3x), dilute aqueous NaOH (3x), water, dried (MgSO<sub>4</sub>) and solvent removed to give **5.42** as a yellow oil (566 mg, 84%), which was not purified further.

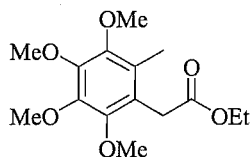
<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 2.30 (s, 3H, ArCH<sub>3</sub>), 3.78 (s, 3H, OCH<sub>3</sub>), 3.85 (s, 3H, OCH<sub>3</sub>), 3.91 (s, 6H, 2x OCH<sub>3</sub>)

<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 15.98, 60.86, 60.90, 61.15, 61.39, 114.32, 127.31, 145.50, 146.35, 147.23, 148.31

HREIMS Found 290.0163 (Calcd. for  $C_{11}H_{15}O_4^{79}Br$  290.0151)

FTIR 2937.4, 2858.3  $cm^{-1}$

**Ethyl 2-(2-methyl-3,4,5,6-tetramethoxyphenyl)acetate (5.44)**

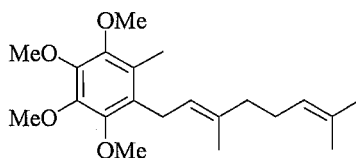


General method A was carried out using **5.42** (475 mg, 1.60 mmol),  $nBuLi$  (1.35 mL of a 1.6M solution in hexane, 1.87 mmol, 1.15 equiv), copper bromide dimethylsulfide complex (235 mg, 1.14 mmol, 0.7 equiv) and ethyl bromoacetate (0.2 mL, 1.79 mmol, 1.1 equiv). Standard work up gave a crude product which, upon purification by radial chromatography eluting with 1:4 ethyl acetate:petroleum ether, afforded **5.44** as a yellow oil (21 mg, 7%).

$^1H$  NMR ( $CDCl_3$ )  $\delta$  1.28 (t,  $J=6.8$  Hz, 3H,  $CH_2CH_3$ ), 2.09 (s, 3H,  $ArCH_3$ ), 3.51 (s, 2H,  $ArCH_2$ ), 3.78 (s, 3H,  $OCH_3$ ), 3.79 (s, 3H,  $OCH_3$ ), 3.89 (s, 3H,  $OCH_3$ ), 3.90 (s, 3H,  $OCH_3$ ), 4.19 (q,  $J=6.8$  Hz, 2H,  $CH_2CH_3$ )

$^{13}C$  NMR ( $CDCl_3$ )  $\delta$  12.05, 14.04, 32.34, 60.61, 60.64, 60.92, 60.96, 61.01, 122.19, 125.94, 144.49, 146.04, 147.70, 148.09, 171.71

**2,6-Dimethyl-8-(2-methyl-3,4,5,6-tetramethoxyphenyl)octa-2,6-diene (5.41)<sup>17</sup>**



General method A was carried out using **5.42** (735 mg, 2.50 mmol) and geranyl bromide (0.55 mL, 2.78 mmol). Upon workup and purification by radial chromatography eluting with 1:19 ethyl acetate:petroleum ether, **5.41** was isolated as a yellow oil (426 mg, 48%).

$^1H$  NMR ( $CDCl_3$ )  $\delta$  1.57 (s, 3H,  $=CCH_3$ ), 1.64 (s, 3H,  $=CCH_3$ ), 1.77 (s, 3H,  $=CCH_3$ ), 2.00 (m, 2H,  $=CCH_2$ ), 2.07 (m, 2H,  $=CCH_2CH_2$ ), 2.14 (s, 3H,  $ArCH_3$ ), 3.32 (d,  $J=6.8$

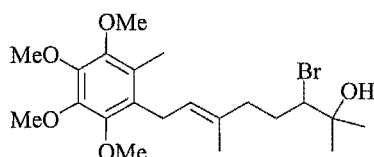
Hz, 2H, ArCH<sub>2</sub>), 3.78 (s, 3H, OCH<sub>3</sub>), 3.79 (s, 3H, OCH<sub>3</sub>), 3.90 (s, 3H, OCH<sub>3</sub>), 3.91 (s, 3H, OCH<sub>3</sub>), 5.04 (m, 2H, 2x CH=)

<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 11.61, 16.11, 17.61, 25.62, 25.73, 26.55, 39.64, 60.61, 61.01, 61.03, 107.01, 122.88, 124.23, 125.35, 129.23, 131.25, 134.93, 144.63, 144.87, 147.61, 147.80

HREIMS Found 348.2306 (Calcd for C<sub>21</sub>H<sub>32</sub>O<sub>4</sub> 348.2301)

FTIR 2931.6, 2860.2, 1467.7 cm<sup>-1</sup>

**3-Bromo-2,6-dimethyl-8-(2-methyl-3,4,5,6-tetramethoxyphenyl)oct-6-en-2-ol  
(5.51)**



General method B was carried out using **5.41** (420 mg, 1.20 mmol) and *N*-bromosuccinimide (236 mg, 1.33 mmol, 1.1 equiv). Work up and purification by radial chromatography, eluting with 1:9 ethyl acetate:petroleum ether to 1:4 ethyl acetate:petroleum ether afforded the bromohydrin **5.51** as a yellow oil (347 mg, 65%).

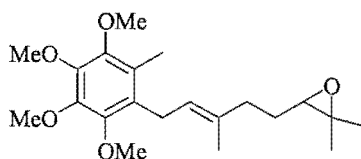
<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.32 (s, 3H, C(OH)CH<sub>3</sub>) 1.33 (s, 3H, C(OH)CH<sub>3</sub>), 1.77 (s, 3H, =CCH<sub>3</sub>), 1.79 (m, 1H, CH<sub>2a</sub>CBr), 1.98 (m, 2H, CH<sub>2b</sub>CBr), 2.09 (m, 1H, =CCH<sub>2a</sub>), 2.35 (m, 1H, =CCH<sub>2b</sub>), 2.14 (s, 3H, ArCH<sub>3</sub>), 3.33 (m, 2H, ArCH<sub>2</sub>), 3.79 (s, 3H, OCH<sub>3</sub>), 3.78 (s, 3H, OCH<sub>3</sub>), 3.90 (s, 3H, OCH<sub>3</sub>), 3.91 (s, 3H, OCH<sub>3</sub>), 3.95 (dd, *J*=1.5 and 11.7 Hz, 1H, CHBr), 5.13 (t, *J*=6.4 Hz, 1H, CH=)

<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 11.78, 16.14, 25.81, 25.86, 26.62, 32.10, 38.23, 60.70 (x2), 61.10 (x2), 70.85, 72.44, 124.42, 125.28, 128.85, 133.24, 144.70, 145.00, 147.62, 147.87

HREIMS found 444.1516 (Calcd for C<sub>21</sub>H<sub>33</sub>O<sub>5</sub><sup>79</sup>Br 444.1509)

FTIR 3477.4, 2935.5, 1739.7 cm<sup>-1</sup>

**3-[3-Methyl-5-(2-methyl-3,4,5,6-tetramethoxyphenyl)pent-3-enyl]-2,2-dimethyloxirane (5.52)**<sup>12</sup>



General method C was carried out using potassium carbonate (2.17 g, 0.02 mol, 70 equiv) and **5.51** (100 mg, 0.22 mmol). Work up afforded **5.52** as a pale yellow oil (64 mg, 78%), with no further purification.

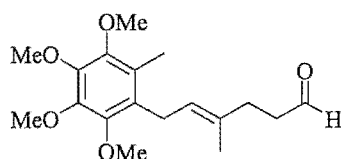
<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.24 (s, 3H, CCH<sub>3</sub>), 1.25 (s, 3H, CCH<sub>3</sub>), 1.64 (m, 2H, CH<sub>2</sub>CO), 1.80 (s, 3H, =CCH<sub>3</sub>), 2.10 (m, 2H, =CCH<sub>2</sub>), 2.17 (s, 3H, ArCH<sub>3</sub>), 2.67 (t, *J*=6.4 Hz, 1H, CHO), 3.32 (d, *J*=2.9 Hz, 2H, ArCH<sub>2</sub>), 3.78 (s, 3H, OCH<sub>3</sub>), 3.79 (s, 3H, OCH<sub>3</sub>), 3.90 (s, 3H, OCH<sub>3</sub>), 3.91 (s, 3H, OCH<sub>3</sub>), 5.10 (t, *J*=5.4 Hz, 1H, CH=)

<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 11.62, 16.07, 18.59, 24.67, 25.67, 27.26, 36.19, 58.17, 60.51, 60.90, 60.93, 63.99, 123.37, 125.12, 128.79, 133.94, 144.57, 144.88, 147.50, 147.73

HREIMS found 364.2254 (Calcd for C<sub>21</sub>H<sub>32</sub>O<sub>5</sub> 364.2250)

FTIR 2960.5, 2933.5, 2862.2, 2829.4 cm<sup>-1</sup>

**4-Methyl-6-(2-methyl-3,4,5,6-tetramethoxyphenyl)hex-4-enal (5.50)**



General method D was carried out using periodic acid (75 mg, 0.33 mmol, 1 equiv) and **5.52** (120 mg, 0.33 mmol). Work up gave **5.50** as a pale yellow oil (90 mg, 85%), with no further purification.

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.79 (s, 3H, =CCH<sub>3</sub>), 2.12 (s, 3H, ArCH<sub>3</sub>), 2.33 (t, *J*=7.8 Hz, 2H, CH<sub>2</sub>CH<sub>2</sub>CHO), 2.52 (dt, *J*=2.0 and 7.3 Hz, 2H, CH<sub>2</sub>CHO), 3.32 (d, *J*=5.9 Hz, 2H, ArCH<sub>2</sub>), 3.78 (s, 3H, OCH<sub>3</sub>), 3.79 (s, 3H, OCH<sub>3</sub>), 3.90 (s, 3H, OCH<sub>3</sub>), 3.91 (s, 3H, OCH<sub>3</sub>), 5.09 (t, *J*= 6.4 Hz, 1H, CH=), 9.74 (t, *J*= 1.9 Hz, 1H, CHO)

<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 11.72, 16.27, 25.76, 31.79, 42.13, 60.68 (x2), 61.07 (x2), 123.99,



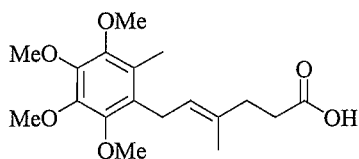
125.27, 128.62, 133.00, 144.68, 145.07, 147.62, 147.85, 202.47

HREIMS Found 322.1771 (Calcd. for  $C_{18}H_{26}O_5$  322.1780)

Anal. Calcd for  $C_{18}H_{26}O_5$ : C 67.1; H 8.1. Found: C 66.9; H 7.9.

FTIR 2935.5, 1724.2  $cm^{-1}$

**4-Methyl-6-(2-methyl-3,4,5,6-tetramethoxyphenyl)hex-4-enoic acid (5.53)<sup>14</sup>**



To a solution of silver nitrate (82 mg, 0.54 mmol, 1.5 equiv) in ethanol (3.9 mL) stirring at room temperature under a nitrogen atmosphere, was added a solution of **5.50** (115 mg, 0.36 mmol) in ethanol (2.3 mL). To this was added dropwise a solution of NaOH (0.18 mL of a 5N aqueous solution) in ethanol (2.3 mL) over a period of 10 min. The resulting black solution was stirred at room temperature for 16 h. The reaction was filtered and ethanol removed under reduced pressure. Water was added, and the solution acidified and extracted with ethyl acetate (2x). Combined organic fractions were dried ( $MgSO_4$ ) and solvent removed to afford **5.53** as a yellow oil (94 mg, 78%), which was not purified further.

$^1H$  NMR ( $CDCl_3$ )  $\delta$  1.79 (s, 3H,  $=CCH_3$ ), 2.12 (s, 3H,  $ArCH_3$ ), 2.31 (t,  $J=7.3$  Hz, 2H,  $=CCH_2$ ), 2.45 (t,  $J=6.8$  Hz, 2H,  $CH_2CO_2H$ ), 3.32 (d,  $J=6.4$  Hz, 2H,  $ArCH_2$ ), 3.77 (s, 3H,  $OCH_3$ ), 3.78 (s, 3H,  $OCH_3$ ), 3.89 (s, 3H,  $OCH_3$ ), 3.90 (s, 3H,  $OCH_3$ ), 5.09 (t,  $J=6.8$  Hz, 1H,  $CH=$ )

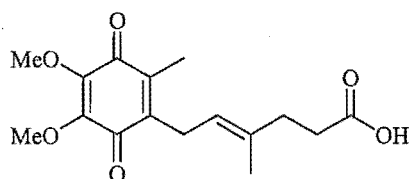
$^{13}C$  NMR ( $CDCl_3$ )  $\delta$  11.69, 16.13, 25.75, 32.67, 34.27, 60.68 (x2), 61.08 (x2), 123.98, 125.32, 128.72, 133.03, 144.65, 145.02, 147.62, 147.83, 178.47

HREIMS Found 338.1729 (Calcd. for  $C_{18}H_{26}O_6$  338.1729)

Anal. Calcd for  $C_{18}H_{26}O_6$ : C 63.9; H 7.7. Found: C 64.1; H 7.8.

FTIR 2935.5, 2659.7, 1708.8  $cm^{-1}$

**4-Methyl-6-(3-methyl-5,6-dimethoxy-1,4-benzoquinon-2-yl)hex-4-enoic acid**  
**(5.37)<sup>14</sup>**



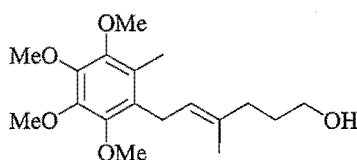
General method G was carried out using ceric ammonium nitrate (365 mg, 0.66 mmol, 2.5 equiv) and **5.53** (90 mg, 0.27 mmol). Work up and purification by radial chromatography eluting with dichloromethane to 1:19 MeOH:dichloromethane gave **5.37** as a bright orange oil (45 mg, 55%).

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.77 (s, 3H, =CCH<sub>3</sub>), 2.00 (s, 3H, ArCH<sub>3</sub>), 2.30 (m, 2H, =CCH<sub>2</sub>), 2.43 (m, 2H, =CCH<sub>2</sub>CH<sub>2</sub>), 3.19 (d, *J*=6.8 Hz, 2H, ArCH<sub>2</sub>), 3.98 (s, 3H, OCH<sub>3</sub>), 3.99 (s, 3H, OCH<sub>3</sub>), 5.00 (m, 1H, CH=)

<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 11.88, 16.13, 25.17, 32.61, 34.19, 61.09 (x2), 120.02, 135.50, 139.01, 141.17, 144.17, 144.27, 179.03, 183.79, 184.60

HREIMS Found 308.1263 (Calcd. For C<sub>16</sub>H<sub>20</sub>O<sub>6</sub> 308.1260)

**4-Methyl-6-(2-methyl-3,4,5,6-tetramethoxyphenyl)hex-4-en-1-ol (5.54)<sup>12</sup>**



General method E was carried out using sodium borohydride (10 mg, 0.27 mmol, 1 equiv) and **5.50** (88 mg, 0.27 mmol). Work up afforded **5.54** as a yellow oil, (65 mg, 74%), which was not further purified.

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.65 (m, 2H, CH<sub>2</sub>CH<sub>2</sub>OH), 1.76 (s, 3H, =CCH<sub>3</sub>), 2.04 (t, *J*=6.8 Hz, 2H, =CCH<sub>2</sub>), 2.11 (s, 3H, ArCH<sub>3</sub>), 3.30 (d, *J*=6.8 Hz, 2H, ArCH<sub>2</sub>), 3.59 (t, *J*=6.4 Hz, 2H, CH<sub>2</sub>OH), 3.76 (s, 3H, OCH<sub>3</sub>), 3.77 (s, 3H, OCH<sub>3</sub>), 3.88 (s, 3H, OCH<sub>3</sub>), 3.89 (s, 3H, OCH<sub>3</sub>), 5.06 (dt, *J*=1.5 and 6.8 Hz, 1H, CH=)

<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 11.88, 16.08, 25.90, 30.77, 36.26, 60.94, 61.99, 61.24 (x2),

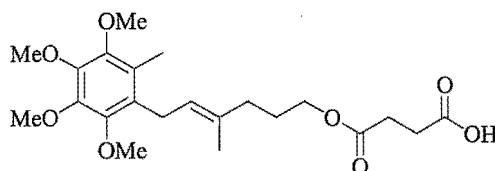
62.76, 123.24, 125.22, 129.03, 134.82, 144.72, 145.03, 147.62, 147.92

HREIMS Found 324.1937 (Calcd. for  $C_{18}H_{28}O_5$  324.1937)

Anal. Calcd for  $C_{18}H_{28}O_5$ : C 66.6; H 8.7. Found: C 66.7; H 8.6.

FTIR 3435.0, 2935.5, 1465.8, 1407.9  $cm^{-1}$

**4-Methyl-6-(2-methyl-3,4,5,6-tetramethoxyphenyl)hex-4-enyl hydrogen succinate (5.55)**



General method F was carried out using succinic anhydride (34 mg, 0.34 mmol, 2.1 equiv), *N,N*-dimethylaminopyridine (1 mg, 0.01 mmol, 0.05 equiv), triethylamine (0.03 mL, 0.24 mmol, 1.5 equiv) and **5.54** (52 mg, 0.16 mmol). Work up and purification by radial chromatography eluting with 1:9 ethyl acetate:petroleum ether afforded **5.55** as a pale yellow oil (24 mg, 37%).

$^1H$  NMR ( $CDCl_3$ )  $\delta$  1.73 (m, 2H,  $CH_2CH_2O$ ), 1.76 (s, 3H,  $=CCH_3$ ), 2.03 (t,  $J=6.8$  Hz, 2H,  $=CCH_2$ ), 2.12 (s, 3H,  $ArCH_3$ ), 2.59 and 2.65 (m, 4H,  $COCH_2CH_2CO_2H$ ), 3.30 (d,  $J=6.4$  Hz, 2H,  $ArCH_2$ ), 3.78 (s, 3H,  $OCH_3$ ), 3.78 (s, 3H,  $OCH_3$ ), 3.89 (s, 3H,  $OCH_3$ ), 3.90 (s, 3H,  $OCH_3$ ), 4.05 (dt,  $J=2.4$  and 6.8 Hz, 2H,  $CH_2O$ ), 5.05 (t,  $J=6.4$  Hz, 1H,  $CH=$ )

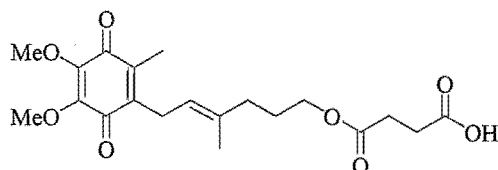
$^{13}C$  NMR ( $CDCl_3$ )  $\delta$  11.66, 15.98, 25.71, 26.19, 26.69, 28.87, 35.65, 60.65, 61.05, 64.46, 64.66, 123.57, 125.32, 126.98, 128.92, 133.71, 144.59, 144.90, 147.48, 147.78, 172.22, 176.96

HREIMS Found 424.2129 (Calcd. For  $C_{22}H_{32}O_8$  424.2097)

Anal. Calcd for  $C_{22}H_{32}O_8$ : C 62.3; H 7.6. Found: C 62.4; H 7.7.

FTIR 2937.4, 1737.7  $cm^{-1}$

**4-Methyl-6-(3-methyl-5,6-dimethoxy-1,4-benzoquinon-2-yl)hex-4-enyl hydrogen succinate (5.38)**



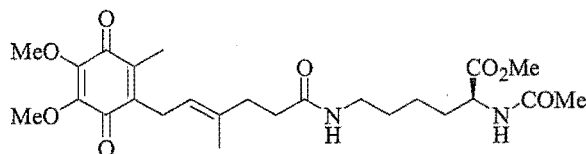
General method G was carried out using **5.55** (24 mg, 0.06 mmol) and ceric ammonium nitrate (77 mg, 0.14 mmol, 2.5 equiv). Work up and purification by radial chromatography eluting with dichloromethane to 1:19 MeOH:dichloromethane gave **5.38** as an orange oil (20 mg, 90%).

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.72 (m, 2H,  $\text{CH}_2\text{CH}_2\text{O}$ ), 1.74 (s, 3H,  $=\text{CCH}_3$ ), 2.02 (s, 3H,  $\text{ArCH}_3$ ), 2.03 (m, 2H,  $=\text{CCH}_2$ ), 2.60-2.66 (m, 4H,  $\text{COCH}_2\text{CH}_2\text{CO}_2\text{H}$ ), 3.18 (d,  $J=6.8$  Hz, 2H,  $\text{ArCH}_2$ ), 3.98 (s, 3H,  $\text{OCH}_3$ ), 4.00 (s, 3H,  $\text{OCH}_3$ ), 4.03 (t,  $J=6.3$  Hz, 2H,  $\text{CH}_2\text{O}$ ), 4.95 (t,  $J=7.1$  Hz, 1H,  $\text{CH}=\text{)$

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  11.95, 16.12, 25.32, 26.58, 28.79, 28.82, 35.66, 61.14 (x2), 64.29, 119.73, 136.27, 138.94, 141.38, 144.21, 144.34, 172.13, 177.24, 183.93, 184.74

HREIMS Found 394.1621 (Calcd. for  $\text{C}_{20}\text{H}_{26}\text{O}_8$  394.1623)

**Methyl 2-acetyl-amino-6-[4-methyl-6-(3-methyl-5,6-dimethoxy-1,4-benzoquinon-2-yl)hex-4-enoylamino]hexanoate (5.58)**



General method H was carried out using **5.37** (10 mg, 0.032 mmol) and *N*- $\alpha$ -acetyl-lysine methyl ester hydrochloride (8.5 mg, 0.036 mmol, 1.1 equiv). Work up and purification by radial chromatography eluting with 1:19 MeOH:dichloromethane, afforded **5.41** as an orange oil (10 mg, 63%).

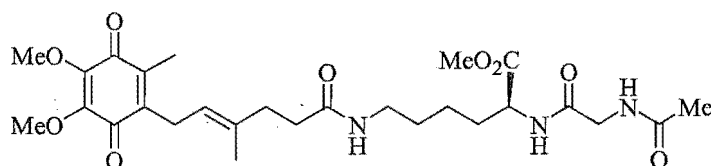
$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.35 (m, 2H,  $\text{NHCH}_2\text{CH}_2$ ), 1.50 (m, 2H,  $\text{NHCH}_2\text{CH}_2\text{CH}_2$ ), 1.68 (m, 1H,  $\text{CH}_{2a}\text{CHCO}_2\text{Me}$ ), 1.76 (s, 3H,  $=\text{CCH}_3$ ), 1.82 (m, 1H,  $\text{CH}_{2b}\text{CHCO}_2\text{Me}$ ), 2.01

(s, 3H, ArCH<sub>3</sub>), 2.04 (s, 3H, COCH<sub>3</sub>), 2.24 (m, 2H, =CCH<sub>2</sub>), 2.29 (m, 2H, =CCH<sub>2</sub>CH<sub>2</sub>), 3.18 (d, *J*=6.8 Hz, 2H, ArCH<sub>2</sub>), 3.21 (m, 2H, NHCH<sub>2</sub>), 3.74 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.99 (s, 3H, OCH<sub>3</sub>), 4.00 (s, 3H, OCH<sub>3</sub>), 4.55 (m, 1H, CHCO<sub>2</sub>Me), 4.99 (t, *J*=7.3 Hz, 1H, CH=), 5.77 (brs, 1H, NH), 6.40 (d, *J*=6.8 Hz, 1H, NH)

<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 11.95, 16.23, 22.25, 23.05, 25.33, 28.84, 31.79, 35.13, 35.27, 38.68, 51.82, 52.38, 61.13, 61.16, 119.91, 136.23, 139.06, 141.24, 144.20, 170.30, 172.85, 172.98, 182.30, 183.90

HRESMS Found 492.2465 (Calcd. for C<sub>25</sub>H<sub>36</sub>N<sub>2</sub>O<sub>8</sub> 492.2472)

**Methyl 2-(2-acetyl-amino-acetyl-amino)-6-[4-methyl-6-(3-methyl-5,6-dimethoxy-1,4-benzoquinon-2-yl)hex-4-enoylamino]hexanoate (5.59)**



General method H was carried out using **5.37** (10 mg, 0.03 mmol) and *N*-acetyl-glycyls methyl ester acetate (11 mg, 0.04 mmol, 1.1 equiv). Purification by radial chromatography eluting with 1:19 MeOH:dichloromethane afforded **5.42** as an orange oil (6 mg, 34%).

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.34 (m, 2H, NHCH<sub>2</sub>CH<sub>2</sub>), 1.46 (m, 2H, NHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 1.71 (m, 1H, CH<sub>2a</sub>CHCO<sub>2</sub>Me), 1.76 (s, 3H, =CCH<sub>3</sub>), 1.85 (m, 1H, CH<sub>2b</sub>CHCO<sub>2</sub>Me), 2.01 (s, 3H, ArCH<sub>3</sub>), 2.05 (s, 3H, COCH<sub>3</sub>), 2.28 (m, 2H, =CCH<sub>2</sub>), 3.15 (m, 1H, NHCH<sub>2a</sub>), 3.17 (d, *J*=7.3 Hz, ArCH<sub>2</sub>), 3.23 (m, 1H, NHCH<sub>2b</sub>), 3.74 (s, 3H, CO<sub>2</sub>CH<sub>3</sub>), 3.91 (dd, *J*=5.4 and 16.1 Hz, 1H, COCH<sub>2a</sub>NH), 3.99 (s, 3H, OCH<sub>3</sub>), 4.00 (s, 3H, OCH<sub>3</sub>), 4.01 (dd, *J*=5.4 and 16.1 Hz, 1H, COCH<sub>2b</sub>NH), 4.53 (m, 1H, CHCO<sub>2</sub>Me), 4.98 (t, *J*=6.8 Hz, CH=), 6.03 (brs, 1H, NH), 6.74 (brs, 1H, NH), 7.08 (d, *J*=7.3 Hz, 1H, NH)

<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 11.97, 16.28, 22.17, 22.89, 25.37, 28.86, 31.21, 35.04, 35.23, 38.55, 43.13, 52.01, 52.45, 61.15, 61.18, 119.85, 136.27, 139.10, 141.25, 143.01, 144.32, 169.08, 170.91, 172.51, 173.01, 183.96, 184.65

HRESMS Found 549.2677 (Calcd. For C<sub>27</sub>H<sub>39</sub>O<sub>9</sub>N<sub>3</sub> 549.2686)

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